MALARIA IMMUNOGEN AND VACCINE

Description

CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation-in-part of application Serial No. 60/225,813, filed August 16, 2000, whose disclosures are incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to the intersection of the fields of immunology and protein engineering, and particularly to an immunogen and vaccine useful in prevention of malaria infection by *P. falciparum* or *P. vivax*.

BACKGROUND OF THE INVENTION

Malaria is by far the world's most important tropical parasitic disease, killing more people than any other communicable disease, with the exception of tuberculosis. The causative agents in humans are four species of *Plasmodium* protozoa: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Although *P. falciparum* accounts for the majority of infections and is responsible for the vast majority of deaths attributable to malaria, *P. vivax* causes a recurring chronic debilitating disease for which a vaccine is necessary.

Malaria infection begins when a female *Anopheles* mosquito infected with one of the four *Plasmodium* species infectious for humans bites a person. The mosquito's saliva carries the malarial sporozoites into the blood. Approximately 30 minutes later these sporozoites enter the liver. Once in the liver, the sporozoites divide over the

course of about 5 days, forming a schizont. A schizont may contain up to 30,000 merozoites, which spill into the bloodstream when the schizont ruptures. Within seconds, merozoites infect red blood cells (RBCs) and again replicate asexually, with each schizont producing up to 36 merozoites.

Each time a RBC bursts and liberates progeny, other blood cells are infected. The cycle can continue until the person dies of anemia and/or other complications. A few of the merozoites in RBCs differentiate into gametocytes, a sexual form, which, if ingested by a mosquito, are liberated from the RBCs in the mosquito stomach and subsequently mate. The progeny, sporozoites, accumulate in the saliva and the process starts again when the mosquito feeds [See, Hoffman et al., (1996) "Attacking the Infected Hepatocyte", in *Malaria Vaccine Development* (ed. S.L. Hoffman), p.35. ASM Press, Washington, D.C., for review).

P. vivax malaria is most prevalent in Latin America (where it is as or more prevalent than *P. falciparum*) and Asia. Although rarely fatal, *P. vivax* malaria has a dormant liver phase that is associated with relapses that show variability in duration, depending on the strain.

Presently, there is not an effective vaccine against any form of malaria. For many years, chloroquine was a cheap and effective therapeutic for treating malaria. But in recent years, chloroquine resistance has increased dramatically for *P. falciparum*. In the past 10 years, *P. vivax* has also developed chloroquine resistance with cases being reported in south-east Asia, the south-west Pacific; Burma [Marlar, T., et al., *Trans. R. Soc. Trop. Med. Hyg.*, 1995. **89**(3): p. 307-308] perhaps India [Garg et al., Trans. R. Soc Trop. Med. Hyg., 1995. **89**(6):

p. 656-657],Indonesia [Baird et al., *Am. J. Trop. Med.
Hyg.*, 1991, **44**(5): p. 547-552; Baird et al., *Trans R Soc
Trop Med Hyg*, 1996, **90**(4): p. 409-411; Baird et al., J.
Infect. Dis., (1995) **171**(6): p. 1678-1682; Murphy et al.,
Lancet, (1993) **341**(8837): p. 96-100; and Schwartz et al.,
[letter]. N. Engl. J. Med., (1991) **324**(13): p. 927] and
Papua New Guinea [Rieckmann et al., *Lancet*, (1989)
2(8673): p. 1183-1184].

Primaquine is the only antimalarial drug that is
effective against hyponozoites, which are associated with
the dormant phase in the liver responsible for relapses.
Different *P. vivax* strains show differential patterns of
relapse; for example, a Korean *P. vivax* strain has been
shown to be 100 percent radically cured by a given
primaquine regime (WHO, 1967), whereas the same regime is
only 70 percent effective with the Chesson strain [Coatney
et al., *J. Natl. Malaria Soc.*, 1962. **9**: p. 285-292]. To
complicate treatment with primaquine further, reports in
the 1970s highlighted primaquine-resistant P.vivax in
south-east Asia [Charoenlarp et al., *Southeast Asian J.
Trop. Med. Public Health*, (1973) **4**(1): p. 135-137 and
Krotoski, *[letter]*. N. Engl. J. Med., (1980) **303**(10): p.
587]; observations have steadily increased in other
locations in recent years [Schuurkamp et al., *Trans. R.
Soc. Trop. Med. Hyg.*, (1992) **86**(2): p. 121-122] suggesting
that widespread resistance to primaquine is emerging.

Clearly, the most effective approach to
combating malaria is an effective vaccine. As with
smallpox, and potentially polio in the near future, a
coordinated worldwide vaccination program can result in
eradication of communicable human diseases. This may also
be achievable for malaria if an effective vaccine can be
developed.

There are three recognized anti-parasitic approaches to malaria vaccine development. These are proposed to function by interrupting the parasite's lifecycle at three different stages.

The first and most attractive approach is the pre-erythrocytic vaccine, which aims to block sporozoite entry into the hepatocyte and/or release of merozoites into the blood stream. Immediately following infection, sporozoites migrate to the liver and begin the exoerythrocytic stage of their lifecycle. Successful blocking of hepatocyte entry, or the destruction of infected hepatocytes prior to liberation of merozoites, would prevent the disease, the passage of the parasite on to feeding mosquitoes, and merozoite release and subsequent invasion of red blood cells.

A second approach is to develop an 'antidisease' vaccine. The target is the red blood cell stage of the infection, during which the parasite grows at an exponential rate. Also known as 'asexual blood stage' vaccines, the merozoite surface protein 1 (MSP-1) and apical membrane antigen 1 (AMA-1) protein have emerged as the two most promising vaccine candidates for intervening at this stage of the disease (See, Good, et al., *Anu. Rev. Immunol.*, (1998) 16: p. 57-87,, for review). This stage is thought to represent a conceptually more difficult target compared with the pre-erythrocytic stage, which is associated with 10-20 sporozoites per mosquito bite, due to the tremendous increase in parasite load once the blood stage is reached.

A third approach, known as the 'transmission-blocking' vaccine, would not stop infection or symptoms in the individual. However, it would prevent infection from spreading to others by blocking the lifecycle in the mosquito by inducing antibodies that the mosquito would

ingest from the host with its blood meal. This vaccine
approach is more attractive as a long-term global solution
to eradication of malaria and less attractive to the
immediate needs of travelers and the military forces.

In the 1960s, researchers at New York University
(NYU) achieved full protection from malaria infection by
injecting animals with small numbers of sporozoites from
mosquitoes that had previously been irradiated. Later,
researchers at the University of Maryland, NYU and Walter
Reed Army Institute showed that percent of a group of
human volunteers immunized with irradiated sporozoites
later resisted exposure to virulent sporozoites [Clyde *et
al.*, *Am. J. Med. Sci.*, (1973) **266**(6): p. 398-403 and
Rieckmann *et al.*, *Trans. R. Soc. Trop. Med. Hyg.*, (1974)
68(3): p. 258-259]. This work confirmed that protective
immunity to the sporozoite stage (i.e. the pre-
erythrocytic stage) of the malaria parasite could be
induced. However, an inability to culture sporozoites *in
vitro* thwarted the possibility of using them as a vaccine.

The strategic development of a synthetic malaria
vaccine required the identification of immunodominant,
neutralizing malaria epitopes. In 1985, a group at NYU
led by Drs. Ruth and Victor Nussenzweig, identified the
dominant B cell epitope from the circumsporozoite protein
(CS), a major component of the sporozoite surface membrane
at the time the parasite enters the bloodstream [Zavala *et
al.*, *Science*, (1985) **228**(4706): p. 1436-40]. Antibodies
to the repeated epitope were shown to be sporozoite
neutralizing by protecting against rodent and human
malaria [Nussenzweig et al., Ciba Found. Symp., (1986)
119: p. 150-163]. Antibodies to the CS protein also
correlated positively with protection in immunized mice
and in naturally infected individuals.

These studies strongly suggest that anti-CS repeat antibodies alone are able to confer protection against malaria infection, provided sufficient antibody titers can be raised. The identification of this epitope therefore enabled, for the first time, the strategic development of synthetic CS-based malaria vaccines. Several malaria vaccine candidates employing different carriers were developed based upon the identification of this epitope. The main focus of malaria vaccine development has been on *P. falciparum*, and it is widely assumed that information gained from studying *P. falciparum* extend to other *Plasmodium* species, including *P. vivax*. A brief overview of four pre-erythrocytic *P. falciparum* malaria vaccine candidates is given below.

The (NANP)$_3$ synthetic peptide conjugated to the protein carrier tetanus toxoid (TT) was the first synthetic malaria vaccine to undergo phase I and phase II clinical trials in the late 1980s [Etlinger et al., *Immunology*, (1988) **64**(3): p. 551-558; Etlinger et al., *J. Immunol.*, (1988) **140**(2): p. 626-633 and Herrington et al., *Nature*, (1987) **328**(6127): p. 257-259]. TT is widely known to provide powerful T cell help for coupled immunogens. Of the thirty-five vaccinees, the three having the highest titers of anti-sporozoite antibodies were selected for challenge studies. One of the vaccine recipients remained free of parasitaemia at 29 days, whereas the other two did not exhibit asexual stage parasites until 11 days, compared with a mean of 8.5 days for the un-vaccinated control group. Therefore, protection again correlated positively with anti-NANP titers.

The limited effectiveness of this vaccine was attributed to suboptimal levels of anti-NANP antibodies. Attempts to increase dosage were hindered by toxicity of the TT carrier. Further, the lack of parasite-derived

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determinants capable of priming malaria-specific T cells also likely contributed to the low levels of protection.

Short synthetic peptides often have an *in vivo* half-life that is too short for them to be effective as prophylactic or therapeutic drugs. Standard approaches for increasing the immunogenicity of peptides is to either couple them to larger carrier proteins, or to assemble them into multimeric structures. In this case, 32 copies of the CS repeat sequence $((NANP)_{15}(NVDP))_2$ were linked and recombinantly fused to a random 32 amino acid fusion protein 20. [Ballou et al., *Lancet*, (1987) 1(8545): p. 1277-1281.] This vaccine candidate was called FSV-1.

Following immunization, twelve of the fifteen volunteers developed antibodies that reacted with sporozoites. No patients exhibited adverse reactions to the protein, indicating that the NANP (SEQ ID NO: 184) repeat itself is non-toxic. Of the fifteen patients immunized with 3 doses, six were selected to receive a fourth dose and were then challenged with the malaria parasite. Parasitaemia did not develop in the volunteer with the highest titer of CS antibodies, and parasitaemia was delayed in two of the other five vaccinees.

As with the NANP-TT vaccine discussed above, protection correlated positively with anti-NANP titers. This vaccine was deemed partially successful in that it reconfirmed that humans can be protected by CS protein subunit vaccines. However, the level of protection was not sufficient to warrant larger trials of this particular candidate.

A major shortfall of this vaccine was that it did not provide an efficient source of T cell help. The only individuals who would have received T cell help from this vaccine would be those in whom the CS repeat served as both a B and T helper (Th) cell epitope. However, this

sequence is known to be a Th epitope for only a limited
number of individuals; i.e. it is highly genetically
restricted.

Nardin and coworkers at NYU have been able
elicit relatively high titers of anti-CS antibody in a
diverse range of genetic backgrounds by combining the NANP
repeat epitope with the T cell site identified by
Berzofsky and Good [Good et al., *Science*, (1987)
235(4792): p. 1059-62] in a MAP format [Calvo-Calle et
al., *J. Immunol.*, (1993) **150**(4): p. 1403-1412]. Using
their proprietary 'universal' form of the CS T cell
epitope, Nardin and co-workers have been able to elicit
anti-CS antibodies in all genetic backgrounds tested,
suggesting that genetic restriction is alleviated by
inclusion of this epitope.

Although MAPs have proven to be excellent
research tools, providing valuable insight into immune
recognition of the CS protein, there are several intrinsic
problems associated with using them in a commercial
vaccine. Their commercial utility has yet to be
established relative to manufacturing and cost issues.
Nevertheless, ongoing human clinical testing of these
vaccine candidates will provide very useful information
pertaining to the actual anti-NANP titers necessary for
protective immunity.

One of the most promising malaria vaccines of
recent times utilizes the hepatitis B surface antigen
(HBsAg) to deliver CS epitopes, an approach developed by
SmithKline Beecham (SKB) that is disclosed in U.S. Patent
No. 5,928,902 that issued on July 27, 1999. That patent
inter alia discloses a hybrid protein comprised of all of
the C-terminal portion of the CS protein, four or more
tandem repeats of the CS immunodominant region and the
hepatitis B surface antigen. The CS epitopes include the

NANP repeat, in concert with additional CS epitopes, including the T cell site identified by Berzofsky and Good [Good et al., *Science*, (1987) **235**(4792): p. 1059-62] (but not the universal form developed by Nardin and co-worker [Moreno et al., *Int. Immunol.*, (1991) **3**(10): p. 997-1003 and Calvo-Calle et al., *J. Immunol.*, (1997) **159**(3): p. 1362-1373]), fused to the hepatitis B surface protein.

This vaccine was recently the subject of human clinical trials [Stoute et al., *N. Engl. J. Med.*, [1997] **336**(2): p. 86-91]. When administered with one of three different adjuvants, this vaccine protected 1/7, 2/7 and 6/7 individuals, respectively. Of the seven individuals immunized with vaccine 2 (adjuvant: oil-in-water emulsion), none of the five patients with anti-CS titers (IFA) in the range of 100-12,800 were protected, whereas the two vaccine recipients with antibody titers in the range of 25,600-51,200 were both protected. Again, protection was correlated positively with anti-CS titers. It is interesting to note that the one patient that received vaccine 1, the alum/oil-in-water formulation, remained protected for at least six months.

The preliminary efficacy report for SKB's malaria vaccine candidate (RTS,S) [Stoute et al. (1997) *N. Engl. J. Med.*, **336**(2): p. 86-91] although encouraging, was tempered by the lack of long-term protection in follow-up studies [Stoute et al. (1998 Oct) *J.infect Dis.*, **178**(4):1139-44]. It was also apparent that the use of a potent and complex adjuvant (SBAS2) containing the immunostimulants QS-21 and monophosphoryl lipid A (MPL), formulated in an oil-in-water emulsion, was essential to achieve efficacy, because volunteers receiving the vaccine formulated on alum were not protected. Five of six patients, who were initially protected after

administration of the RTS,S/SBAS2 formulation, were not protected six months after receiving the third vaccine dose. Similar results were recently reported at the 48th annual meeting of the American Society of Tropical Medicine and Hygiene for a field trial conducted in Africa.

Like HBsAg, HBcAg is a particulate protein derived from the hepatitis B virus that has been proposed as a carrier for heterologous epitopes. The relative immunogenicity of HBsAg (HBs) has been compared with HbcAg (HBc), and the ability of each to evoke immune responses in different genetic backgrounds [Milich et al., *Science*, (1986) **234**(4782): p. 1398-1401]. These data emphasize the higher immunogenicity of HBc relative to HBs, and the universal responsiveness to HBc, irrespective of genetic background.

For example, HBc is more than 300 times more immunogenic than HBs in BALB/c mice; and, although both B10.S and B10.M mice are non-responders to HBs, every strain tested is responsive to HBc. These results re-emphasize the suitability of HBc as a vaccine carrier and specifically, its superiority over HBs, hence the selection of HBc as opposed to HBs to carry heterologous epitopes. These facets of HBc are thought to be particularly important in malaria vaccine development, because they address the genetic restriction and inadequate antibody titers that have been largely responsible for the inability to develop an effective vaccine using the neutralizing CS epitopes.

The positive correlation between protection against malaria infection and anti-CS antibody titer has been demonstrated repeatedly over the past 15 years [Etlinger et al., *Immunology*, (1988) 64(3): p. 551-558; Etlinger et al., *J. Immunol.*, (1988) 140(2): p. 626-633;

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Ballou et al., *Lancet*, (1987) 1(8545): p. 1277-1281; Stoute et al., *N. Engl. J. Med.*, (1997) 336(2): p. 86-91 and Herrington *et al.,. Am J Trop Med Hyg,* (1991) 45(6): p. 695-701]. The evidence that a vaccine eliciting high-titer, long-lived antibody responses in sufficient vaccine recipients can be protective suggests that protection against malaria infection is achievable via anti-sporozoite antibody production.

Using rodent models of malaria, it has been found that malaria CS-repeats fused to the immunodominant loop of HBc were able to protect mice against both *P. berghei* and, perhaps more impressively, *P. yoelii* to levels of 90-100 percent [Schodel et al., *Behring Inst. Mitt.*, 1997(98): p. 114-119 and Schodel et al., *J. Exp. Med.*, (1994) **180**(3): p. 1037-46]. Further, antibody responses to the *P. berghei* particle were shown to prime antibody responses effectively over a wide range of genetic backgrounds, confirming the universal priming effects of HBc [Schodel et al., *J. Exp. Med.*, (1994) **180**(3): p. 1037-46].

Another advantage of the HBc carrier is the fact that it does not require complex adjuvants for efficacy. This is due to the high inherent immunogenicity of the particle. A comparison of the immunogenicity of HBc-*P. berghei* particles showed that alum, which is approved for human use, was more effective than either IFA or CFA [Schodel et al., *J. Exp. Med.*, (1994) **180**(3): p. 1037-46]. The importance of this observation is highlighted by toxicity problems associated with newer, more complex adjuvants as was recently noted in clinical trials of SKB's candidate malaria vaccine [Stoute et al., *N. Engl. J. Med.*, [1997] **336**(2): p. 86-91].

The immunodominant B cell epitope of the CS protein of *P. falciparum*, which has been more widely studied than *P. vivax*, is a highly conserved repeated tetrapeptide (NANP) [Zavala *et al.*, *Science*, (1985) **228**(4706): p. 1436-40], and antibodies to this epitope have been shown to be sporozoite-neutralizing in protecting against rodent and human malaria. Immune responsiveness to this epitope has been positively correlated with immunity to malaria in both vaccine recipients and naturally infected individuals. Indeed, a review of clinical trials data for pre-erythrocytic vaccines described previously (HBs-CS, FSV-1, NANP-TT), highlights a strong correlation between antibody titer and protection. Those individuals who have been protected by previous vaccine candidates have been associated with the highest anti-NANP antibody titers, with the possible exception of SKB's candidate vaccine (# 3 - RTS,S and adjuvant SBAS2 containing MPL and QS-21 in a water-in-oil formulation) where adjuvants appeared to play a critical role in protection, because protection was not long-lived, as noted before.

The family hepadnaviridae are enveloped DNA-containing animal viruses that can cause hepatitis B in humans (HBV). The hepadnavirus family includes hepatitis B viruses of other mammals, e.g., woodchuck (WHV), and ground squirrel (GSHV), and avian viruses found in ducks (DHV) and herons (HeHV). Hepatitis B virus (HBV) used herein refers to a member of the family hepadnaviridae, unless the discussion is referring to a specific example.

The nucleocapsid or core of the mammalian hepatitis B virus (HBV or hepadnavirus) contains a

sequence of 183 or 185 amino acid residues, depending
on viral subtype, whereas the duck virus capsid
contains 262 amino acid residues. Hepatitis B core
protein monomers self-assemble into stable aggregates
known as hepatitis B core protein particles (HBc
particles). Two three-dimensional structures are
reported for HBc particles. A first that comprises a
minor population contains 90 copies of the HBc
subunit protein as dimers or 180 individual monomeric
proteins, and a second, major population that
contains 120 copies of the HBc subunit protein as
dimers or 240 individual monomeric proteins. These
particles are referred to as T = 4 or T = 3
particles, respectively, wherein "T" is the
triangulation number. These human HBc particles are
about are about 30 or 34 nm in diameter,
respectively. Pumpens et al., (1995) *Intervirology*,
38:63-74; and Metzger et al., (1998) *J. Gen. Viol.*,
79:587-590. See also, Wynne et al., (June 1999) *Mol.
Cell*, **3**:771-780.

Conway et al., (1997) *Nature*, **386**:91-94,
describe the structure of human HBc particles at 9
Ångstrom resolution, as determined from cryo-electron
micrographs. Bottcher et al. (1997), *Nature*, **386**:88-
91, describe the polypeptide folding for the human
HBc monomers, and provide an approximate numbering
scheme for the amino acid residues at which alpha
helical regions and their linking loop regions form.
Zheng et al. (1992), *J. Biol. Chem.*, **267(13)**:9422-
9429 report that core particle formation is not
dependent upon the arginine-rich C-terminal domain,
the binding of nucleic acids or the formation of
disulfide bonds based on their study of mutant
proteins lacking one or more cysteines and others'

work with C-terminal-truncated proteins [Birnbaum et al., (1990) *J.Virol*. **64**, 3319-3330].

The nucleocapsid or viral core protein (HBc) has been disclosed as an immunogenic carrier moiety that stimulates the T cell response of an immunized host animal. See, for example, U.S. Patents No. 4,818,527, No 4,882,145 and No. 5,143,726. A particularly useful application of this carrier is its ability to present foreign or heterologous B cell epitopes at the site of the immunodominant loop that is present at about residue positions 70-90, and more usually recited as about positions 75 through 85 from the amino-terminus (N-terminus) of the protein. Clarke et al. (1991) F. Brown et al. eds., *Vaccines 91*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp.313-318.

During viral replication, HBV nucleocapsids associate with the viral RNA pre-genome, the viral reverse transcriptase (Pol), and the terminal protein (derived from Pol) to form replication competent cores. The association between the nucleocapsid and the viral RNA pre-genome is mediated via an arginine-rich domain at the carboxyl-terminus (C-terminus). When expressed in heterologous expression systems, such as *E.coli* where viral RNA pre-genome is absent, the protamine-like C-terminus; i.e., residues at positions 150 through 183, binds *E.coli* RNA.

In an application as a vaccine carrier moiety, it is preferable that the HBV nucleocapsids not bind nucleic acid derived from the host. Birnbaum et al., (1990) *J.Virol*. **64**, 3319-3330 showed that the protamine-like C-terminal domain of HBV nucleocapsids could be deleted without interfering with the protein's ability to assemble into virus-

like particles. It is thus reported that proteins truncated to about position 144; i.e., containing the HBc sequence from position one through about 144, can self-assemble, whereas deletions beyond residue 139 abrogate capsid assembly [Seifer et al., (1995) Intervirology, **38**:47-62].

More recently, Metzger et al., (1998) *J. Gen. Viol.*, **79**:587-590 reported that the proline at position 138 (Pro-138 or P138) of the human sequence is required for particle formation. Those authors also reported that assembly capability of particles truncated at the carboxy-terminus to lengths of 142 and 140 residues was affected, with assembly capability being completely lost with truncations resulting in lengths of 139 and 137 residues.

Several groups have shown that truncated particles exhibit reduced stability relative to standard hepatitis B core particles [Gallina et al. (1989) *J.Virol.*, **63**:4645-4652; Inada, et al. (1989) *Virus Res.*, **14**:27-48], evident by variability in particle sizes and the presence of particle fragments in purified preparations [Maassen et al. (1994) *Arch. Virol.*, **135**:131-142]. Thus, prior to the report of Metzger et al., above, Pumpens et al., (1995) *Intervirology*, **38**:63-74 summarized the literature reports by stating that the carboxy-terminal border for HBc sequences required for self-assembly was located between amino acid residues 139 and 144, and that the first two or three amino-terminal residues could be replaced by other sequences, but elimination of four or eleven amino-terminal residues resulted in the complete disappearance of chimeric protein in transformed *E. coli* cells.

Recombinantly-produced hybrid particles bearing internal insertions (referred to in the art as HBc chimeric particles or HBc chimers) often appear to have a less ordered structure, when analyzed by electron microscopy, compared to particles that lack heterologous epitopes [Schodel et al., (1994) *J.Exp.Med.*, **180**:1037-1046]. In some cases the insertion of heterologous epitopes into C-terminally truncated HBc particles has such a dramatic destabilizing affect that hybrid particles cannot be recovered following heterologous expression [Schodel et al. (1994) *Infect. Immunol.*, **62**:1669-1676]. Thus, many chimeric HBc particles are so unstable that they fall apart during purification to such an extent that they are unrecoverable or they show very poor stability characteristics, making them problematic for vaccine development.

Chimeric hepatitis B core particles have been prepared by heterologous expression in a wide variety of organisms, including *E.coli*, *B.subtilis*, *Vaccinia*, *Salmonella typhimurium*, *Saccharomyces cerevisiae*. See, for example Pumpens et al., (1995) *Intervirology*, **38**:63-74 , and the citations therein that note the work of several research groups, other than the present inventors.

A structural feature whereby the stability of full-length HBc particles could be retained, while abrogating the nucleic acid binding ability of full-length HBc particles, would be highly beneficial in vaccine development using the hepadnaviral nucleocapsid delivery system. Indeed, Ulrich et al. in their recent review of the use of HBc chimers as carriers for foreign epitopes [*Adv. Virus Res.*, **vol.50** (1998) Academic Press pages 141-182] note

three potential problems to be solved for use of those chimers in human vaccines. A first potential problem is the inadvertent transfer of nucleic acids in a chimer vaccine to an immunized host. A second potential problem is interference from preexisting immunity to HBc. A third possible problem relates to the requirement of reproducible preparation of intact chimer particles that can also withstand long-term storage.

Initial evaluation of a particle displaying epitopes from *P. falciparum* [CS-2; Schodel et al., *J. Exp. Med.*, (1994) **180**(3): p. 1037-46] was encouraging. However, using that particle as an immunogen in a vaccine in mice, provided antibody titers that were lower than those observed for the *P. berghei* and *P. yoelii* particles.

There are recognized to be two main CS-repeat epitopes associated with *P. vivax* (type-I and type-II), and a third, reported in 1993 and called 'vivax-like', which is identical to the CS-repeat from the monkey parasite (*P. siminovale*) resembling *P. ovale* [Qari et al., *Lancet*, 1993. **341**(8848): p. 780-783]. For simplicity, this CS-repeat is referred to herein as *P. vivax* type-III.

The benefits of the inclusion of a universal T (Th) cell epitope derived from the malaria parasite are several-fold. First, the priming of malaria-specific Th cells ensures that, should a vaccine recipient be exposed to malaria, a more rapid and stronger anti-malaria response is activated due to previous priming of malaria specific T-helper cells. Secondly, vaccinees living in malaria endemic regions experience natural 'boosting' every time they are exposed to the parasite, because their immune systems have been primed at both the B and Th cell level. This effect is similar to clinical boosting by re-

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vaccination, a process that can be difficult to coordinate in developing countries where malaria is endemic.

Although the CS gene is largely invariant, limited sequence variation has been noted to occur mainly in the immunodominant T cell epitopic domains. The fact that genetic mutations always appear to result in amino acid substitutions suggests that pressure at the protein level, possibly immunological pressure, has selected for variation. Typically, the problems associated with amino acid variability of an epitope can only be resolved by the inclusion of multiple variants of the epitope. However, Nardin and coworkers at New York University recently identified a consensus form of the T cell epitope CS 326-345 that appears to bind all class II MHC molecules [Calvo-Calle et al., *J. Immunol.*, (1993) **150**(4): p. 1403-1412 and Moreno et al., *J. Immunol.*, (1993) **151**(1): p. 489-499].

Studies have shown that this consensus epitope is 'universal', like the T cell help afforded by HBc, and suggests that it primes malaria-specific Th cells in essentially all vaccine recipients. The fact that this epitope of the CS protein was identified by CD4$^+$ T cells of volunteers that were protected against malaria following exposure to irradiated sporozoites, confirms that it is efficiently processed and presented *in vivo* by antigen presenting cells (APC) when presented in the context of sporozoite [Moreno et al., *Int. Immunol.*, (1991) **3**(10): p. 997-1003]. The identification of this epitope was a significant advancement in the task of developing a pre-erythrocytic stage malaria vaccine.

As disclosed hereinafter, the present invention provides a contemplated HBc chimer that provides unexpectedly high titers of antibodies against malaria sporozoites, and in one aspect also

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provides a solution to the problems of HBc chimer
stability as well as the substantial absence of
nucleic acid binding ability of the construct. In
addition, a contemplated recombinant chimer exhibits
minimal, if any, antigenicity toward preexisting
anti-HBc antibodies.

BRIEF SUMMARY OF THE INVENTION

The present invention contemplates an
immunogen for inducing antibodies to the malaria-
causing parasite, *Plasmodium*, and particularly the
species P. falciparum and *P. vivax*, and a vaccine
comprising that immunogen dispersed in a
physiologically tolerable diluent. A contemplated
immunogen is a recombinant hepatitis B virus core
(HBc) protein chimer molecule with a length of about
140 to about 310 amino acid residues that contains
four peptide-linked amino acid residue sequence
domains from the N-terminus that are denominated
Domains I, II, III and IV.

The first domain, Domain I, comprises about
71 to about 85 amino acid residues whose sequence
includes at least the sequence of the residues of
position 5 through position 75 of HBc.

The second domain, Domain II, comprises
about 18 to about 58 amino acid residues peptide-
bonded to residue 75 of which (i) a sequence of HBc
is present from HBc positions 76 through 85 and (ii)
a sequence of 8 to about 48 residues that constitute
a B cell epitope of the CS protein of a species of
the parasite *Plasmodium* that is peptide-bonded
between the HBc residues of positions 78 and 79.

The third domain, Domain III, is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85.

The fourth domain, Domain IV, comprises (i) zero to fourteen residues of a HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues, (iii) fewer than three arginine or lysine residues, or mixtures thereof adjacent to each other, and (iv) up to about 100 amino acid residues in a sequence heterologous to HBc from position 150 to the C-terminus, with the proviso that at least five amino acid residues are present of the amino acid residue sequence from position 136 through 149; i. e., residues of positions 136-140, when (a) zero cysteine residues are present and (b) fewer than about five heterologous amino acid residues are present, thus, Domain IV contains at least 5 residues.

In preferred embodiments, the immunogen is in the form of self-assembled particles and the *Plasmodium* B cell epitope is that of *P. falciparum* or *P. vivax*. It is also preferred that the HBc sequence of Domain I includes the residues of position 1 through position 75 with no additional residues at the N-terminus. It is further preferred that a contemplated immunogen contain one cysteine residue within Domain IV in an amino acid residue sequence heterologous to that of HBc from position 150 to the C-terminus. It is particularly preferred that that heterologous sequence comprise a T cell epitope from the same species of *Plasmodium* as the B cell epitope.

Another embodiment comprises an inoculum or vaccine that comprises an above HBc chimer particle

that is dissolved or dispersed in a pharmaceutically acceptable diluent composition that typically also contains water. When administered in an immunogenic effective amount to an animal such as a mammal or bird, an inoculum induces antibodies that immunoreact specifically with the chimer particle or the conjugated (pendently-linked) hapten. The antibodies so induced also preferably immunoreact specifically with (bind to) an antigen containing the hapten, such as a protein where the hapten is a peptide or a saccharide where the hapten is an oligosaccharide.

The present invention has several benefits and advantages.

A particular benefit of the invention is that its use as a vaccine provides extraordinary antibody titers against the *Plasmodium* species of the B cell epitope.

An advantage of the invention is that those very high antibody titers have been produced with the aid of an adjuvant approved for use in humans.

Another benefit of the invention is that the recombinant immunogen is prepared easily and using well known cell culture techniques.

Another advantage of the invention is that the immunogen is easily prepared using well known recombinant techniques.

Yet another benefit of the invention is that a preferred immunogen exhibits greater stability at elevated temperatures than to other HBc chimers.

Yet another advantage of the invention is that a contemplated immunogen is substantially free of nucleic acids.

Still further benefits and advantages will be apparent to the worker of ordinary skill from the disclosure that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings forming a portion of this disclosure:

Fig. 1 shows the modifications made to commercial plasmid vector pKK223-3 in the preparation of plasmid vector pKK223-3N used herein for preparation of recombinant HBc chimers. The modified sequence (SEQ ID NO:180) is shown below the sequence of the commercially available vector (SEQ ID NO:181). The bases of the added NcoI site are shown in lower case letters and the added bases are shown with double underlines, whereas the deleted bases are shown as dashes. The two restriction sites present in this segment of the sequence (NcoI and HindIII) are indicated.

Fig. 2, shown in three panels as Figs. 2A, 2B and 2C, schematically illustrates a preferred cloning strategy in which a malarial B cell epitope such as (NANP)$_4$ (SEQ ID NO:1) is cloned into the EcoRI and SacI sites of an engineered HBc gene (Fig. 2A) between positions 78 and 79, which destroys the EcoRI site, while preserving the SacI site. Fig. 2B shows DNA (double stranded and encoded amino acid residue sequence) that encodes a T cell epitope such as that referred to as Pf/CS326-345 (Pf/CS-UTC) and a stop codon (SEQ ID NOs:79 and 80) cloned into the EcoRI and HindIII sites at the C-terminus of an engineered, truncated HBc gene containing the first 149 HBc residues (HBc149). PCR amplification of the construct of Fig. 2B using a primer having a 5'-

terminal SacI restriction site adjacent to a HBc-
encoding sequence beginning at residue position 79
followed by digestion of the amplified sequence and
the construct of Fig. 2A with SacI, followed by
ligation of the appropriate portions is shown in Fig.
2C to form a single gene construct referred to
hereinafter as V12 that encodes a B cell- and T cell-
containing immunogen for a vaccine against *P.
falciparum*.

Fig. 3 is a photograph of an SDS-PAGE
analysis under reducing conditions to show the
stabilizing effects on expressed particles of a codon
for a single cysteine residue inserted in frame
between the C-terminal codon (V149) and the
termination codon of HBc in a chimer that also
contains (NANP)$_4$ inserted between the amino acids of
positions 78 and 79 (V2.Pf1+C), and a similar
construct whose C-terminus is residue V149 (V2.Pf1)
at day zero and after 15 days at 37°C. [Lane 1,
V2.Pf1 - day 0; Lane 2, V2.Pf1 - day 15 at 37°C; Lane
3, V2.Pf1+C, day 0; Lane 4, V2.Pf1+C - day 15 at
37°C.]

Fig. 4 is a photograph of an SDS-PAGE
analysis under reducing conditions that illustrates
the stabilizing effects on chimer HBc149 particles
containing (NANP)$_4$ inserted between amino acids 78
and 79 and the cysteine-containing T cell epitope
fused to the C-terminus [V2.Pf1+Pf/CS-UTC also
referred to as V12.Pf1] as compared to a similar
particle in which the C-terminal Cys was replaced by
an Ala residue [V2.Pf1+ Pf/CS-UTC(C17A) also referred
to as V12.Pf1(C17A)] at day zero and after 28 days at
37°C. [Lane 1, V2.Pf1+Pf/CS-UTC - day zero; Lane 2,

V2.Pf1+ Pf/CS-UTC - day 28 at 37°C; Lane 3,
V2.Pf1+Pf/CS-UTC(C17A) - day zero; Lane 4, V2.Pf1+
Pf/CS-UTC(C17A) - day 28 at 37°C.]

Fig. 5 is a graph showing the results of an
indirect immunofluorescence assay (IFA) carried out
using glutaraldehyde-fixed *P. falciparum* sporozoites
and FITC-labeled anti-mouse IgG (gamma-chain
specific) to detect bound antibody titers (log of
1/dilution; ordinate) over time in weeks (abscissa)
for three chimeric immunogens after immunization in
mice. Data for the prior art chimer immunogen, CS-2,
are shown as squares, those for the recombinant HBc
chimer V12.Pf1 are shown as diamonds, whereas those
for the recombinant HBc chimer V12.Pf3.1 are shown as
triangles.

Fig.6, shown in two panels as Fig. 6A and
Fig. 6B, provides an alignment of six published
sequences for mammalian HBc proteins from six
viruses. The first (SEQ ID NO:170), human viral
sequence is of the *ayw* subtype and was published in
Galibert et al. (1983) *Nature*, **281**:646-650; the
second human viral sequence (SEQ ID NO:171), of the
adw subtype, was published by Ono et al. (1983)
Nucleic Acids Res., **11(6)**: 1747-1757; the third human
viral sequence (SEQ ID NO:172), is of the *adw2*
subtype and was published by Valenzuela et al.,
Animal Virus Genetics, Field et al. eds., Academic
Press, New York (1980)pages 57-70; the fourth human
viral sequence (SEQ ID NO:173), is of the *adyw*
subtype that was published by Pasek et al. (1979)
Nature, **282**:575-579; the fifth sequence (SEQ ID
NO:174), is that of the woodchuck virus that was
published by Galibert et al. (1982) *J. Virol.*, **41**:51-
65; and the sixth mammalian sequence, (SEQ ID

NO:168), is that of the ground squirrel that was
published by Seeger et al. (1984) *J. Virol.*,**51**:367-
375.

Figure 7 is a photograph of an SDS-PAGE
analysis under reducing conditions following
incubations at 37°C for 0, 1 and 2 days that
illustrates the stabilizing effects on (1) chimer
HBc149 particles containing the *P. falciparum* (NANP)$_4$
immunogenic sequence inserted between HBc amino acid
residues 78 and 79 that also contain a carboxy-
terminal universal *P. falciparum* malarial T cell
epitope peptide-bonded to HBc position 149 [UTC;
V12.Pf1 = V2.Pf1 + Pf/CS-UTC], and (2) similar
particles in which the cysteine at position 17 of the
UTC was mutated to be an alanine residue and a
cysteine residue was added at residue position 150,
between the HBc residue at position 149 and the
beginning of the UTC [V12.Pf1(C17A)+C150].
Definitions

Numerals utilized in conjunction with HBc
chimers indicate the position in the HBc *ayw* amino
acid residue sequence of SEQ ID NO:170 at which one
or more residues has been added to the sequence,
regardless of whether additions or deletions to the
amino acid residue sequence are present. Thus,
HBc149 indicates that the chimer ends at residue 149,
whereas HBc149 + C150 indicates that that same chimer
contains a cysteine residue at HBc position 150. On
the other hand, the malarial CS protein universal T
cell epitope (UTC) is 20 residues long, and a
replacement of the cysteine at position 17 in that
sequence by an alanine is referred to as CS-
UTC(C17A).

The term "antibody" refers to a molecule that is a member of a family of glycosylated proteins called immunoglobulins, which can specifically bind to an antigen.

The word "antigen" has been used historically to designate an entity that is bound by an antibody or receptor, and also to designate the entity that induces the production of the antibody. More current usage limits the meaning of antigen to that entity bound by an antibody or receptor, whereas the word "immunogen" is used for the entity that induces antibody production or binds to the receptor. Where an entity discussed herein is both immunogenic and antigenic, reference to it as either an immunogen or antigen is typically made according to its intended utility.

"Antigenic determinant" refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site or T-cell receptor. The term is also used interchangeably with "epitope".

The word "conjugate" as used herein refers to a hapten operatively linked to a carrier protein, as through an amino acid residue side chain.

The term "conservative substitution" as used herein denotes that one amino acid residue has been replaced by another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another such as between arginine and lysine, between glutamic and aspartic acids or between glutamine and asparagine and the like.

The term "corresponds" in its various grammatical forms as used in relation to peptide sequences means the peptide sequence described plus or minus up to three amino acid residues at either or both of the amino- and carboxy-termini and containing only conservative substitutions in particular amino acid residues along the polypeptide sequence.

The term "Domain" is used herein to mean a portion of a recombinant HBc chimer molecule that is identified by (i) residue position numbering relative to the position numbers of HBcAg subtype *ayw* as reported by Galibert et al., (1979) *Nature*, 281:646-650 (SEQ ID NO:170). The polypeptide portions of at least chimer Domains I, II and III are believed to exist in a similar tertiary form to the corresponding sequences of naturally occurring HBcAg.

As used herein, the term "fusion protein" designates a polypeptide that contains at least two amino acid residue sequences not normally found linked together in nature that are operatively linked together end-to-end (head-to-tail) by a peptide bond between their respective carboxy- and amino-terminal amino acid residues. The fusion proteins of the present invention are HBc chimers that induce the production of antibodies that immunoreact with a polypeptide or pathogen-related immunogen that corresponds in amino acid residue sequence to the polypeptide or pathogen-related portion of the fusion protein.

The phrase "hepatitis B" as used here refers in its broadest context to any member of the family hepadnaviridae, as discussed before.

The term "residue" is used interchangeably with the phrase amino acid residue.

DETAILED DESCRIPTION OF THE INVENTION

The present invention contemplates an immunogen and a vaccine comprising that immunogen against the malaria parasite, particularly those that infect humans; i.e., *P. falciparum* and *P. vivax*. Historically, one of the main shortfalls of peptide-based vaccines has been the lack of persistence of antibody following immunization. As discussed hereinafter, using the HBc chimer immunogen applied to *P. falciparum* vaccine development, high titers of neutralizing antibody are maintained for more than 6 months in mice following a 2-dose immunization regimen. This is consistent with and superior to the protection studies in the *P. yoelii* model using a similar but differently constructed immunogen, in which immunity obtained from challenge infection was evident 3 months after immunization using an immunogen different from that used here. [Schodel et al., *Behring Inst. Mitt.*, 1997(98): p. 114-119.]

A contemplated immunogen is a recombinant hepatitis B virus core (HBc) protein/chimer molecule with a length of about 140 to about 310 and preferably about 155 to 235 amino acid residues that contains four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV.

(a) Domain I comprises about 71 to about 85 amino acid residues whose sequence includes at least the sequence of the residues of position 5 through position 75 of HBc.

(b) Domain II comprises about 11 to about 58 amino acid residues peptide-bonded to residue 75. This sequence includes (i) a sequence of HBc from HBc

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positions 76 through 85 and (ii) a sequence of 8 to
about 48 residues that constitute a B cell epitope of
the circumsporozoite protein of a species of the
parasite *Plasmodium* that is peptide-bonded between
the HBc residues of positions 78 and 79.

(c) Domain III is an HBc sequence from
position 86 through position 135 that is peptide-
bonded to residue 85.

d) Domain IV comprises (i) zero to fourteen
residues of a HBc amino acid residue sequence from
position 136 through 149 peptide-bonded to the
residue of position 135 of Domain III, (ii) zero to
three cysteine residues, (iii) fewer than three
arginine or lysine residues, or mixtures thereof
adjacent to each other, and (iv) up to 100, and
preferably up to 25, amino acid residues in a
sequence heterologous to HBc from position 150 to the
C-terminus, with the proviso that at least five amino
acid residues of the amino acid residue sequence from
position 136 through 149; i.e., residues of positions
136-140, are present when (a) zero cysteine residues
are present and (b) fewer than about five
heterologous amino acid residues are present.

In examining the length of a contemplated
HBc chimer, such a recombinant protein can have a
length of about 140 to about 310 amino acid residues.
Preferably, that length is about 155 to about 235
residues. More preferably, the length is about 165
to about 210 residues. Most preferably, the length
is about 190 to about 200 residues. These
differences in length arise from changes in the
length of Domains I, II and IV.

HBc chimers having a Domain I that contains
more than a deletion of the first three amino-

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terminal (N-terminal) residues have been reported to
result in the complete disappearance of HBc chimer
protein in *E. coli* cells. Pumpens et al.,(1995)
Intervirology, 38:63-74. On the other hand, a recent
study in which an immunogenic 23-mer polypeptide from
the influenza M2 protein was fused to the HBc N-
terminal sequence reported that the resultant fusion
protein formed particles when residues 1-4 of the
native HBc sequence were replaced. Neirynck et al.
(October 1999) *Nature Med.*, 5(10):1157-1163. Thus,
the art teaches that particles can form when an added
amino acid sequence is present peptide-bonded the one
of residues 1-5 of HBc, whereas particles do not form
if no additional sequence is present and more than
residues 1-3 are deleted from the N-terminus of HBc.

An N-terminal sequence peptide-bonded to
one of the first five N-terminal residues of HBc can
contain a sequence of up to about 25 residues that
are heterologous to HBc. Exemplary sequences include
a B cell or T cell epitope such as those discussed
hereinafter, a sequence of another (heterologous)
protein such as β-galactosidase as can occur in
fusion proteins as a result of the expression system
used, or another hepatitis B-related sequence such as
that from the Pre-S1 or Pre-S2 regions or the major
HbsAg immunogenic sequence.

Domain I preferably has the sequence of
residues of positions 1 through 75 of HBc, and is
free of added residues at the amino-terminus (N-
terminus). Domain I is also therefore preferably
free of deletions of residues of positions 1-3.

Domain II, which is peptide-bonded to
residue 75, contains the sequence of HBc residues of
positions 76 through 85, and has a malarial B cell

epitope whose length is 8 through about 28 residues peptide-bonded between residues 78 and 79. Preferred malarial B cell epitopes are discussed hereinafter.

Preferred malarial B cell epitopes for insertion between residues 78 and 79 of a recombinant HBc chimer are enumerated in Table A, below.

Table A
Malarial B Cell Epitopes

P. falciparum

$(NANP)_4$	SEQ ID NO:1
$NANPNVDP(NANP)_3NVDP$	SEQ ID NO:2
$NANPNVDP(NANP)_3$	SEQ ID NO:3
$(NANP)_3NVDPNANP$	SEQ ID NO:4
$NANPNVDP(NANP)_3NVDPNANP$	SEQ ID NO:5
$NPNVDP(NANP)_3NV$	SEQ ID NO:6
$NPNVDP(NANP)_3NVDP$	SEQ ID NO:7
$NPNVDP(NANP)_3NVDPNA$	SEQ ID NO:8
$NVDP(NANP)_3NV$	SEQ ID NO:9
$NVDP(NANP)_3NVDP$	SEQ ID NO:10
$NVDP(NANP)_3NVDPNA$	SEQ ID NO:11
$DP(NANP)_3NV$	SEQ ID NO:12
$DP(NANP)_3NVDP$	SEQ ID NO:13
$DP(NANP)_3NVDPNA$	SEQ ID NO:14

P. vivax

DRAAGQPAGDRADGQPAG	SEQ ID NO:15
ANGAGNQPGANGAGDQPGA- NGADNQPGANGADDQPG	SEQ ID NO:16
ANGAGNQPGANGAGDQPG	SEQ ID NO:17
ANGADNQPGANGADDQPG	SEQ ID NO:18

ANGAGNQPGANGADNQPG   SEQ ID NO:19

ANGADNQPGANGADDQPG   SEQ ID NO:20

APGANQEGGAAAPGANQEGGAA   SEQ ID NO:21

P. bergeii

$(DP_4NPN)_2$ SEQ ID NO:22

P. yoelli

$(QGPGAP)_4$ SEQ ID NO:23

Domain III contains the sequence of HBc position 86 through position 135 peptide-bonded at its N-terminus to residue 85.

Domain IV comprises (i) zero to fourteen residues of a HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues, (iii) fewer than three arginine or lysine residues, or mixtures thereof adjacent to each other, and (iv) up to about 100 amino acid residues in a sequence heterologous to HBc from position 150 to the C-terminus (typically as one or more T cell epitopes), with certain provisos. Although Domain IV can contain up to about 100 residues that are heterologous to HBc from position 150 through the C-terminus, this domain needs no residues in addition to those recited before to provide an effective immunogen.

However, when the chimeric protein ends at HBc residue 135, desired, particularly immunogenic particles do not form even when a C-terminal cysteine is present. On the other hand, desired particles do form when residues of positions 136-140 are present with or without an added C-terminal cysteine or when

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(a) one cysteine residue is present and (b) about five heterologous amino acid residues are also present peptide-bonded to HBc residue 135. Put differently, Domain IV can end at HBc residue 135 so long as at least five heterologous residues are present and a cysteine residue is also present. Otherwise, Domain IV ends at least at HBc residue 140. Thus, Domain IV contains at least 5 amino acid residues.

It is preferred that Domain IV contain up to fourteen residues of an HBc sequence from position 136 through position 149 peptide-bonded to residue 135; i.e., an HBc sequence that begins with the residue of position 136 that can continue through position 149. Thus, if the residue of position 148 is present, so is the sequence of residues of positions 136 through 147, or if residue 141 is present, so is the sequence of residues of positions 136 through 140.

In one embodiment, Domain IV comprises a sequence of HBc from residue 136 through 140 peptide-bonded to the residue of position 135 of Domain III. The remainder of Domain IV contains (i) zero to nine residues of a HBc amino acid residue sequence from position 141 through 149 peptide-bonded to the position 136-140 sequence, (ii) zero to three cysteine residues, (iii) fewer than three arginine or lysine residues, or mixtures thereof adjacent to each other, and (iv) up to 50 amino acid residues, and more preferably up to about 25 residues, in a sequence that constitutes a T cell epitope of the same species of *Plasmodium* as the B cell epitope peptide-bonded to the final HBc amino acid residue present in the chimer or a cysteine residue. Thus,

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for example, the T cell epitope can be bonded to the carboxy-terminal-most HBc residue such as residue 149, or to a cysteine residue that is bonded to that final HBc residue.

Domain IV can also contain zero to three cysteine residues and those Cys residues are present within about 30 residues of the carboxy-terminus (C-terminus) of the chimer molecule. Preferably, one cysteine (Cys) residue is present, and that Cys is preferably present as the carboxy-terminal (C-terminal) residue, unless a malarial T cell epitope is present as part of Domain IV. When such a T cell epitope is present, the preferred Cys is preferably within the C-terminal last five residues of the HBc chimer. Preferred malarial T cell epitopes are discussed hereinafter.

The presence of the above-discussed cysteine residue(s) provides an unexpected enhancement of the ability of the chimer molecules to form immunogenic particles, as well as unexpected thermal stability to an immunogen particle (discussed hereinafter). Thus, a preferred HBc chimer immunogen tends to be stable to decomposition at 37°C to a greater extent than does a similar chimer lacking that cysteine residue. This enhanced stability is illustrated in Figs. 3, 4 and 7, and is discussed hereinafter.

Domain IV contains fewer than three arginine or lysine residues, or mixtures thereof adjacent to each other. Arginine and lysines are present in the C-terminal region of HBc that extends from position 150 through the C-terminus of the native molecule. That region is sometimes referred to in the are as the "protamine" or "arginine-rich"

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region of the molecule and is thought to bind to nucleic acids. A contemplated HBc chimer molecule and particle are substantially free of bound nucleic acids.

The substantial freedom of nucleic acid binding can be readily determined by a comparison of the absorbance of the particles in aqueous solution measured at both 280 and 260 nm; i.e., a 280/260 absorbance ratio. The contemplated particles do not bind substantially to nucleic acids that are oligomeric and/or polymeric DNA and RNA species originally present in the cells of the organism used to express the protein. Such nucleic acids exhibit an absorbance at 260 nm and relatively less absorbance at 280 nm, whereas a protein such as a contemplated chimer absorbs relatively less at 260 nm and has a greater absorbance at 280 nm.

Thus, recombinantly expressed HBc particles or chimeric HBc particles that contain the arginine-rich sequence at residue positions 150-183 (or 150-185) exhibit a ratio of absorbance at 280 nm to absorbance at 260 nm (280:260 absorbance ratio) of about 0.8, whereas particles free of the arginine-rich nucleic acid binding region of naturally occurring HBc such as those that contain fewer than three arginine or lysine residues or mixtures thereof adjacent to each other, or those having a native or chimeric sequence that ends at about HBc residue position 140 to position 149, exhibit a 280:260 absorbance ratio of about 1.2 to about 1.6.

Chimeric HBc particles of the present invention are substantially free of nucleic acid binding and exhibit a 280:260 absorbance ratio of about 1.2 to about 1.6, and more typically, about 1.4

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to about 1.6. This range is due in large part to the
number of aromatic amino acid residues present in
Domains II and IV of a given chimeric HBc particle.
That range is also in part due to the presence of the
Cys in Domain IV of a contemplated chimer, whose
presence can diminish the observed ratio by about 0.1
for a reason that is presently unknown.

The contemplated chimer HBc particles are
more stable in aqueous buffer at 37°C over a time
period of about two weeks to about one month than are
particles formed from a HBc chimer containing the
same peptide-linked Domain I, II and III sequences
and an otherwise same Domain IV sequence in which the
one to three cysteine residues [C-terminal cysteine
residue(s)] are absent or a single C-terminal residue
present is replaced by another residue such as an
alanine residue. Stability of various chimer
particles is determined as discussed hereinafter.

Thus, for example, particles containing a
heterologous malarial epitope in Domain II [e.g.
(NANP)$_4$] and a single cysteine residue C-terminal to
residue valine 149 is more stable than otherwise
identical particles assembled from chimer molecules
whose C-terminal residue is valine 149. Similarly,
particles containing the above malarial B cell
epitope in Domain II and the universal malarial T
cell epitope that contains a single cysteine near the
C-terminus are more stable than are otherwise
identical particles in which that cysteine is
replaced by an alanine residue. See, Figs. 3, 4 and
7 and the discussion relating thereto hereinafter.

A contemplated particle containing a C-
terminal cysteine residue is also typically prepared
in greater yield than is a particle assembled from a

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chimer molecule lacking a C-terminal cysteine. This increase in yield can be seen from the mass of particles obtained or from integration of traces from analytical gel filtration analysis using Superose® 6 HR as discussed hereinafter and shown in Tables 9A and 9B.

Although the T cell help afforded by HBc is highly effective in enhancing antibody responses (i.e. B cell-mediated) to 'carried' epitopes following vaccination, HBc does not activate malaria-specific T cells, except in restricted individuals for whom the B cell epitope is also a T cell epitope. To help ensure universal priming of malaria-specific T helper cells, in addition to B cells, one or more malaria-specific T helper epitopes is preferably incorporated into a contemplated immunogen and is located in Domain IV of the immunogen.

A particularly preferred recombinant HBc chimer includes a T cell epitope of the same *Plasmodium* species as the B cell epitope. Thus, where the B cell epitope of Domain II is that of *P. falciparum*, the T cell epitope is also that of *P. falciparum*, and the like. Using this matching strategy, T cells are primed to the same species as that to which antibodies are initially induced by the B cell epitope. Particularly preferred T cell epitopes present as a part of Domain IV are enumerated in Table B, below.

Table B

Malarial Universal T Cell Epitope

P. falciparum

GIEYLNKIQNSLSTEWSPCSVT   SEQ ID NO:24

P. vivax

YLDKVRATVGTEWTPCSVT   SEQ ID NO:25

P. yoelli

EFVKQISSQLTEEWSQCSVT   SEQ ID NO:26

A plurality of the above or another T cell epitopes can be present in Domain IV or another B cell epitope can be present. In preferred practice, Domain IV has up to about 50 residues in a sequence heterologous to HBc. Most preferably, that sequence is up to about 25 residues and includes one of the universal T cell epitopes shown in Table B, above.

A contemplated recombinant HBc chimer molecule is typically present and is used in an immunogen or vaccine as a self-assembled particle. These particles are comprised of 180 to 240 chimer molecules that separate into protein molecules in the presence of disulfide reducing agents such as 2-mercaptoethanol, and the individual molecules are therefore thought to be bound together into the particle primarily by disulfide bonds. These particles are similar to the particles observed in patients infected with HBV, but these particles are non-infectious. Upon expression in various prokaryotic and eukaryotic hosts, the individual recombinant HBc chimer molecules assemble in the host

into particles that can be readily harvested from the host cells.

The amino acid sequence of HBc from residue position 1 through at least position 140 is preferably present in a contemplated chimer molecule and particle. The sequence from position 1 through position 149 is more preferably present. A malarial B cell epitope is present between residues 78 and 79 and a single cysteine residue or a malarial T cell epitope containing a cysteine residue is preferably present as a C-terminal addition to the HBc sequence as part of Domain IV. A contemplated recombinant HBc chimer is substantially free of bound nucleic acid. A preferred chimer particle that contains an added Cys residue at or near the C-terminus of the molecule is also more stable at 37°C than is a similar particle that does not contain that added Cys.

In addition to the before-discussed N- and C-truncations and insertion of malarial epitopes, a contemplated chimer molecule can also contain conservative substitutions in the amino acid residues that constitute HBc Domains I, II, III and IV. Conservative substitutions are as defined before.

More rarely, a "nonconservative" change, e.g., replacement of a glycine with a tryptophan is contemplated. Analogous minor variations can also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs well known in the art, for example LASERGENE software (DNASTAR Inc., Madison, Wis.)

The HBc portion of a chimer molecule of the present invention [the portion having the HBc

sequence that has other than a sequence of an added
epitope, or heterologous residue(s) that are a
restriction enzyme artifact] most preferably has the
amino acid residue sequence at positions 1 through
149 of subtype *ayw* that is shown in Fig. 6 (SEQ ID
NO:170), when present. Somewhat less preferred are
the corresponding amino acid residue sequences of
subtypes *adw*, *adw2* and *adyw* that are also shown in
Fig. 6 (SEQ ID NOs:171, 172 and 173). Less preferred
still are the sequences of woodchuck and ground
squirrel at aligned positions 1 through 149 that are
the last two sequences of Fig 6 (SEQ ID NOs:174 and
168). As noted elsewhere, portions of different
sequences from different mammalian HBc proteins can
be used together in a single chimer.

When the HBc portion of a chimer molecule
of the present invention has other than a sequence of
a mammalian HBc molecule at positions 1 through 149,
when present, because one or more conservative
substitutions has been made, it is preferred that no
more than 10 percent, and more preferably no more
than 5 percent, and most preferably no more than 3
percent of the amino acid residues are substituted as
compared to SEQ ID NO:170 from position 1 through
149. A contemplated chimer of 149 HBc residues can
therefore contain up to about 15 residues that are
different from those of SEQ ID NO:170 at positions 1
through 149, and preferably about 7 or 8 residues.
More preferably, up to about 5 residues are different
from the *ayw* sequence (SEQ ID NO:170) at residue
positions 1-149. Where a HBc sequence is truncated
further at one or both termini, the number of
substituted residues is proportionally different.
Deletions elsewhere in the molecule are considered

conservative substitutions for purposes of
calculation.

Chimer Preparation

A contemplated chimeric immunogen is
prepared using the well known techniques of
recombinant DNA technology. Thus, sequences of
nucleic acid that encode particular polypeptide
sequences are added and deleted from the precursor
sequence that encodes HBV.

As was noted previously, the HBc
immunodominant loop is usually recited as being
located at about positions 75 through 85 from the
amino-terminus (N-terminus) of the intact protein.
The malarial B cell epitope-containing sequence is
placed into that immunodominant loop sequence of
Domain II. That placement substantially eliminates
the HBc immunogenicity and antigenicity of the HBc
loop sequence, while presenting the malarial B cell
epitope in an extremely immunogenic position in the
assembled chimer particles.

One of two well-known strategies is
particularly useful for placing the malarial B cell
sequence into the loop sequence at the desired
location between residues 78 and 79. A first, less
successful strategy is referred to as replacement in
which DNA that codes for a portion of the loop is
excised and replaced with DNA that encodes a malarial
B cell sequence. The second strategy is referred to
as insertion in which a malarial B cell sequence is
inserted between adjacent residues in the loop.

Site-directed mutagenesis using the
polymerase chain reaction (PCR) is used in one
exemplary replacement approach to provide a chimeric

HBc DNA sequence that encodes a pair of different
restriction sites, e.g. EcoRI and SacI, one near each
end of the immunodominant loop-encoding DNA.
Exemplary residues replaced are 76 through 81. The
loop-encoding section is excised, a desired malarial
B cell epitope-encoding sequence flanked on each side
by appropriate HBc sequence residues is ligated into
the restriction sites and the resulting DNA is used
to express the HBc chimer. See, for example, Table 2
of Pumpens et al., (1995) *Intervirology*, **38**:63-74 for
exemplary uses of a similar technique.

Alternatively, a single restriction site or
two sites can be encoded into the region, the DNA cut
with a restriction enzyme(s) to provide "sticky" or
ends, and an appropriate sticky- or blunt-ended
heterologous DNA segment ligated into the cut region.
Examples of this type of sequence replacement into
HBc can be found in the work reported in Schodel et
al., (1991) F. Brown et al. eds., *Vaccines 91*, Cold
Spring Harbor Laboratory, Cold Spring Harbor, New
York, pp.319-325, Schodel et al., *Behring Inst.
Mitt.*, 1997(98): p. 114-119 and Schodel et al., *J.
Exp. Med.*, (1994) **180**(3): p. 1037-4, the latter two
papers discussing the preparation of vaccines against
P. yoelii and *P. berghei*, respectively.

It has surprisingly been found that the
insertion position within the HBc immunogenic loop
and the presence of loop residues are of import to
the activity of the immunogen. Thus, as is
illustrated hereinafter, placement of a malarial B
cell epitope between HBc residue positions 78 and 79
provides a particulate immunogen that is ten to one
thousand times more immunogenic than placement of the
same immunogen in an excised and replaced region

between residues 76 and 81. In addition, placement
of the same malarial immunogen between residues 78
and 79 as compared to between residues 77 and 78
provided an unexpected enhancement of about 15-fold.
Thus, a replacement strategy that results in a net
removal of residues from the immunodominant loop is
not used herein.

Insertion is therefore preferred. In an
illustrative example of the insertion strategy, site-
directed mutagenesis is used to create two
restriction sites adjacent to each other and between
codons encoding adjacent amino acid residues, such as
those at residue positions 78 and 79. This technique
adds twelve base pairs that encode four amino acid
residues (two for each restriction site) between
formerly adjacent residues in the HBc loop.

Upon cleavage with the restriction enzymes,
ligation of the DNA coding for the malarial sequence
and expression of the DNA to form HBc chimers, the
HBc loop amino acid sequence is seen to be
interrupted on its N-terminal side by the two
residues encoded by the 5' restriction site, followed
toward the C-terminus by the malarial B-cell epitope
sequence, followed by two more heterologous, non-loop
residues encoded by the 3' restriction site and then
the rest of the loop sequence. This same strategy is
also preferably used for insertion into Domain IV of
a T cell epitope or one or more cysteine residues
that are not a part of a T cell epitope. A similar
strategy using an insertion between residues 82 and
83 is reported in Schoedel et al., (1990) F. Brown et
al. eds., *Vaccines 90*, Cold Spring Harbor Laboratory,
Cold Spring Harbor, New York, pp.193-198.

More specifically, this cloning strategy is illustrated schematically in Figs. 2A, 2B and 2C. In Fig. 2A, a DNA sequence that encodes a C-terminal truncated HBc sequence (HBc149) is engineered to contain adjacent EcoRI and SacI sites between residues 78 and 79. Cleavage of that DNA with both enzymes provides one fragment that encodes HBc positions 1-78 3'-terminated with an EcoRI sticky end, whereas the other fragment has a 5'-terminal SacI sticky end and encodes residues of positions 79-149. Ligation of a synthetic nucleic acid having a 5' AATT overhang followed by a sequence that encodes a desired malarial B cell epitope and a AGCT 3'overhang provides a HBc chimer sequence that encodes that B cell epitope flanked on each side by two heterologous residues (GI and EL, respectively) between residues 78 and 79, while destroying the EcoRI site and preserving the SacI site.

A similar strategy is shown in Fig. 2B for insertion of a cysteine-containing sequence, such as a particularly preferred T cell epitope such as that referred to asPF/CS326-345 (Pf-UTC). Here, EcoRI and HindIII restriction sites were engineered in to the HBc DNA sequence after amino acid residue position 149. After digestion with EcoRI and HindIII, a synthetic DNA having the above AATT 5'overhang followed by a T cell epitope-encoding sequence, a stop codon and a 3' AGCT overhang were ligated into the digested sequence to form a sequence that encoded HBc residues 1-149 followed by two heterologous residues (GI), the stop codon and the HindIII site.

PCR amplification using a forward primer having a SacI restriction site followed by a sequence encoding HBc beginning at residue position 79,

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followed by digestion with SacI and HindIII provided a sequence encoding HBc positions 79-149 plus the two added residues and the T cell epitope at the C-terminus. Digestion of the construct of Fig. 2B with SacI and ligation provided the complete gene encoding a desired recombinant HBc chimer immunogen having the sequence, from the N-terminus, of HBc positions 1-78, two added residues, the malarial B cell epitope, two added residues, HBc positions 79-149, two added residues, and the T cell epitope that is shown in Fig. 2C.

It is noted that the preferred use of two heterologous residues on either side of (flanking) a B cell or T cell epitope is a matter of convenience. As a consequence, one can also use zero to three or more added residues that are not part of the HBc sequence on either or both sides of an inserted sequence. One or both ends of the insert and HBc nucleic acid can be "chewed back" with an appropriate nuclease (e.g. S1 nuclease) to provide blunt ends that can be ligated together. Added heterologous residues that are neither part of the inserted B cell or T cell epitopes nor a part of the HBc sequence are not counted in the number of residues present in a recited Domain.

It is also noted that one can also synthesize all or a part of a desired recombinant HBc chimer nucleic acid using well-known synthetic methods as is discussed and illustrated in U. S. Patent No.5,656,472 for the synthesis of the 177 base pair DNA that encodes the 59 residue ribulose bis-phosphate carboxylase-oxygenase signal peptide of *Nicotiana tabacum*. For example, one can synthesize Domains I and II with a blunt or "sticky" end that

can be ligated to Domains III and IV to provide a construct that expresses a contemplated HBc chimer that contains zero added residues to the N-terminal side of the B cell epitope and zero to three added residues on the C-terminal side or at the Domain II/III junction or at some other desired location.

A nucleic acid sequence (segment) that encodes a previously described HBc chimer molecule or a complement of that coding sequence is also contemplated herein. Such a nucleic acid segment is present in isolated and purified form in some preferred embodiments.

In living organisms, the amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the gene that codes for the protein. Thus, through the well-known degeneracy of the genetic code additional DNAs and corresponding RNA sequences (nucleic acids) can be prepared as desired that encode the same chimer amino acid residue sequences, but are sufficiently different from a before-discussed gene sequence that the two sequences do not hybridize at high stringency, but do hybridize at moderate stringency.

High stringency conditions can be defined as comprising hybridization at a temperature of about 50°-55°C in 6XSSC and a final wash at a temperature of 68°C in 1-3XSSC. Moderate stringency conditions comprise hybridization at a temperature of about 50°C to about 65°C in 0.2 to 0.3 M NaCl, followed by washing at about 50°C to about 55°C in 0.2X SSC, 0.1% SDS (sodium dodecyl sulfate).

A nucleic sequence (DNA sequence or an RNA sequence) that (1) itself encodes, or its complement

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encodes, a chimer molecule whose HBc portion from residue position 1 through 136, when present, is that of SEQ ID NOs: 168, 170, 171, 172, 173 or 174 and (2) hybridizes with a DNA sequence of SEQ ID NOs: 169, 175, 176, 177, 178 or 179 at least at moderate stringency (discussed above); and (3) whose HBc sequence shares at least 80 percent, and more preferably at least 90 percent, and even more preferably at least 95 percent, and most preferably 100 percent identity with a DNA sequence of SEQ ID NOs: 169, 175, 176, 177, 178 and 179, is defined as a DNA variant sequence. As is well-known, a nucleic acid sequence such as a contemplated nucleic acid sequence is expressed when operatively linked to an appropriate promoter in an appropriate expression system as discussed elsewhere herein.

An analog or analogous nucleic acid (DNA or RNA) sequence that encodes a contemplated chimer molecule is also contemplated as part of this invention. A chimer analog nucleic acid sequence or its complementary nucleic acid sequence encodes a HBc amino acid residue sequence that is at least 80 percent, and more preferably at least 90 percent, and most preferably is at least 95 percent identical to the HBc sequence portion from residue position 1 through residue position 136 shown in SEQ ID NOs: 168, 170, 171, 172, 173 and 174. This DNA or RNA is referred to herein as an "analog of" or "analogous to" a sequence of a nucleic acid of SEQ ID NOs: 169, 175, 176, 177, 178 and 179, and hybridizes with the nucleic acid sequence of SEQ ID NOs: 169, 175, 176, 177, 178 and 179 or their complements herein under moderate stringency hybridization conditions. A nucleic acid that encodes an analogous sequence, upon

suitable transfection and expression, also produces a contemplated chimer.

Different hosts often have preferences for a particular codon to be used for encoding a particular amino acid residue. Such codon preferences are well known and a DNA sequence encoding a desired chimer sequence can be altered, using *in vitro* mutagenesis for example, so that host-preferred codons are utilized for a particular host in which the enzyme is to be expressed. In addition, one can also use the degeneracy of the genetic code to encode the HBc portion of a sequence of SEQ ID NOs: 168, 170, 171, 172, 173 or 174 that avoids substantial identity with a DNA of SEQ ID Nos: 169, 175, 176, 177, 178 or 179, or their complements. Thus, a useful analogous DNA sequence need not hybridize with the nucleotide sequences of SEQ ID NOs: 169, 175, 176, 177, 178 or 179 or a complement under conditions of moderate stringency, but can still provide a contemplated chimer molecule.

A recombinant nucleic acid molecule such as a DNA molecule, comprising a vector operatively linked to an exogenous nucleic acid segment (e.g., a DNA segment or sequence) that defines a gene that encodes a contemplated chimer, as discussed above, and a promoter suitable for driving the expression of the gene in a compatible host organism, is also contemplated in this invention. More particularly, also contemplated is a recombinant DNA molecule that comprises a vector comprising a promoter for driving the expression of the chimer in host organism cells operatively linked to a DNA segment that defines a gene for the HBc portion of a chimer or a DNA variant that has at least 90 percent identity to the chimer

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gene of SEQ ID NOs: 169, 175, 176, 177, 178 or 179
and hybridizes with that gene under moderate
stringency conditions.

Further contemplated is a recombinant DNA
molecule that comprises a vector containing a
promoter for driving the expression of a chimer in
host organism cells operatively linked to a DNA
segment that is an analog nucleic acid sequence that
encodes an amino acid residue sequence of a HBc
chimer portion that is at least 80 percent identical,
more preferably 90 percent identical, and most
preferably 95 percent identical to the HBc portion of
a sequence of SEQ ID NOs: 168, 170, 171, 172, 173 or
174. That recombinant DNA molecule, upon suitable
transfection and expression in a host cell, provides
a contemplated chimer molecule.

It is noted that because of the 30 amino
acid residue N-terminal sequence of ground squirrel
HBc does not align with any of the other HBc
sequences, that sequence and its encoding nucleic
acid sequences and their complements are not included
in the above percentages of identity, nor are the
portions of nucleic acid that encode that 30-residue
sequence or its complement used in hybridization
determinations. Similarly, sequences that are
truncated at either or both of the HBc N- and C-
termini are not included in identity calculations,
nor are those sequences in which residues of the
immunodominant loop are removed for insertion of a
heterologous epitope. Thus, only those HBc-encoding
bases or HBc sequence residues that are present in a
chimer molecule are included and compared to an
aligned nucleic acid or amino acid residue sequence
in the identity percentage calculations.

Inasmuch as the coding sequences for the gene disclosed herein is illustrated in SEQ ID NOs: 169, 175, 176, 177, 178 and 179, isolated nucleic acid segments, preferably DNA sequences, variants and analogs thereof can be prepared by *in vitro* mutagenesis, as is well known in the art and discussed in Current Protocols In Molecular Biology, Ausabel et al. eds., John Wiley & Sons (New York: 1987) p. 8.1.1-8.1.6, that begin at the initial ATG codon for a gene and end at or just downstream of the stop codon for each gene. Thus, a desired restriction site can be engineered at or upstream of the initiation codon, and at or downstream of the stop codon so that other genes can be prepared, excised and isolated.

As is well known in the art, so long as the required nucleic acid, illustratively DNA sequence, is present, (including start and stop signals), additional base pairs can usually be present at either end of the segment and that segment can still be utilized to express the protein. This, of course, presumes the absence in the segment of\ an operatively linked DNA sequence that represses expression, expresses a further product that consumes the enzyme desired to be expressed, expresses a product that consumes a wanted reaction product produced by that desired enzyme, or otherwise interferes with expression of the gene of the DNA segment.

Thus, so long as the DNA segment is free of such interfering DNA sequences, a DNA segment of the invention can be about 500 to about 15,000 base pairs in length. The maximum size of a recombinant DNA molecule, particularly an expression vector, is governed mostly by convenience and the vector size

that can be accommodated by a host cell, once all of
the minimal DNA sequences required for replication
and expression, when desired, are present. Minimal
vector sizes are well known. Such long DNA segments
are not preferred, but can be used.

DNA segments that encode the before-
described chimer can be synthesized by chemical
techniques, for example, the phosphotriester method
of Matteucci et al. (1981) *J. Am. Chem. Soc.*,
103:3185. Of course, by chemically synthesizing the
coding sequence, any desired modifications can be
made simply by substituting the appropriate bases for
those encoding the native amino acid residue
sequence. However, DNA segments including sequences
discussed previously are preferred.

A contemplated HBc chimer can be produced
(expressed) in a number of transformed host systems,
typically host cells although expression in
acellular, *in vitro*, systems is also contemplated.
These host cellular systems include, but are not
limited to, microorganisms such as bacteria
transformed with recombinant bacteriophage, plasmid,
or cosmid DNA expression vectors; yeast transformed
with yeast expression vectors; insect cell systems
infected with virus expression vectors (e.g.
baculovirus); plant cell systems transformed with
virus expression vectors (e.g. cauliflower mosaic
virus; tobacco mosaic virus) or with bacterial
expression vectors (e.g., Ti plasmid); or
appropriately transformed animal cell systems such as
CHO or COS cells. The invention is not limited by
the host cell employed.

DNA segments containing a gene encoding the
HBc chimer are preferably obtained from recombinant

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DNA molecules (plasmid vectors) containing that gene. Vectors capable of directing the expression of a chimer gene into the protein of a HBc chimer is referred to herein as an "expression vector".

An expression vector contains expression control elements including the promoter. The chimer-coding gene is operatively linked to the expression vector to permit the promoter sequence to direct RNA polymerase binding and expression of the chimer-encoding gene. Useful in expressing the polypeptide coding gene are promoters that are inducible, viral, synthetic, constitutive as described by Poszkowski et al. (1989) *EMBO J.*, **3**:2719 and Odell et al. (1985) *Nature*, **313**:810, as well as temporally regulated, spatially regulated, and spatiotemporally regulated as given in Chua et al. (1989) *Science*, **244**:174-181.

One preferred promoter for use in prokaryotic cells such as *E. coli* is the Rec 7 promoter that is inducible by exogenously supplied nalidixic acid. A more preferred promoter is present in plasmid vector JHEX25 (available from Promega) that is inducible by exogenously supplied isopropyl-β-D-thiogalacto-pyranoside (IPTG). A still more preferred promoter, the tac promoter, is present in plasmid vector pKK223-3 and is also inducible by exogenously supplied IPTG. The pKK223-3 plasmid can be successfully expressed in a number of *E. coli* strains, such as XL-1, TB1, BL21 and BLR, using about 25 to about 100 μM IPTG for induction. Surprisingly, concentrations of about 25 to about 50 μM IPTG have been found to provide optimal results in 2 L shaker flasks and fermentors.

Several strains of *Salmonella* such as *S. typhi* and *S. typhimurium and S. typhimurium-E. coli*

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hybrids have been used to express immunogenic transgenes including prior HBc chimer particles both as sources of the particles for use as immunogens and as live, attenuated whole cell vaccines and inocula, and those expression and vaccination systems can be used herein. See, U.S. Patent No. 6,024,961; U.S. Patent No. 5,888,799; U.S. Patent No. 5,387,744; U.S. Patent No. 5,297,441; Ulrich et al., (1998) *Adv. Virus Res.*, **50**:141-182; Tacket et al., (Aug 1997) *Infect. Immun.*, **65(8)**:3381-3385; Schodel et al., (Feb 1997) *Behring Inst. Mitt.,* **98**:114-119; Nardelli-Haefliger et al., (Dec 1996) *Infect. Immun.*, **64(12)**:5219-5224; Londono et al., (Apr 1996) *Vaccine,* **14(6)**:545-552, and the citations therein.

Expression vectors compatible with eukaryotic cells, such as those compatible with yeast cells or those compatible with cells of higher plants or mammals, are also contemplated herein. Such expression vectors can also be used to form the recombinant DNA molecules of the present invention. Vectors for use in yeasts such as *S. cerivisiae* or *Pichia pastoris* can be episomal or integrating, as is well known. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Normally, such vectors contain one or more convenient restriction sites for insertion of the desired DNA segment and promoter sequences. Optionally, such vectors contain a selectable marker specific for use in eukaryotic cells. Exemplary promoters for use in *S. cerevisiae* include the *S. cerevisiae* phosphoglyceric acid kinase (PGK) promoter and the divergent promoters GAL 10 and GAL 1, whereas the alcohol oxidase gene (AOX1) is a useful promoter for *Pichia pastoris*.

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For example, to produce chimers in the methylotrophic yeast, *P. pastoris*, a gene that encodes a desired chimer is placed under the control of regulatory sequences that direct expression of structural genes in *Pichia*. The resultant expression-competent forms of those genes are introduced into *Pichia* cells.

More specifically, the transformation and expression system described by Cregg et al. (1987) *Biotechnology*, **5**:479-485; (1987) *Molecular and Cellular Biology*, **12**:3376-3385 can be used. A gene for a chimer V12.Pf3.1 is placed downstream from the alcohol oxidase gene (AOX1) promoter and upstream from the transcription terminator sequence of the same AOX1 gene. The gene and its flanking regulatory regions are then introduced into a plasmid that carries both the *P. pastoris* HIS4 gene and a *P. pastoris* ARS sequence (Autonomously Replicating Sequence), which permit plasmid replication within *P. pastoris* cells [Cregg et al. (1987) *Molecular and Cellular Biology*, **12**:3376-3385].

The vector also contains appropriate portions of a plasmid such as pBR322 to permit growth of the plasmid in *E. coli* cells. The resultant plasmid carrying a chimer gene, as well as the various additional elements described above, is illustratively transformed into a his4 mutant of *P. pastoris*; i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

After selecting transformant colonies on media lacking histidine, cells are grown on media lacking histidine, but containing methanol as described Cregg et al. (1987) *Molecular and Cellular Biology*, **12**:3376-3385, to induce the AOX1 promoters.

The induced AOX1 promoters cause expression of the chimer protein and the production of chimer particles in *P. pastoris*.

A contemplated chimer gene can also be introduced by integrative transformation, which does not require the use of an ARS sequence, as described by Cregg et al. (1987) *Molecular and Cellular Biology*, **12**:3376-3385.

Production of chimer particles by recombinant DNA expression in mammalian cells is illustratively carried out using a recombinant DNA vector capable of expressing the chimer gene in Chinese hamster ovary (CHO) cells. This is accomplished using procedures that are well known in the art and are described in more detail in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratories (1989).

In one illustrative example, the simian virus (SV40) based expression vector, pKSV-10 (Pharmacia Fine Chemicals, Piscataway, NJ), is subjected to restriction endonuclease digestion by NcoI and HindIII. A NcoI/HindIII sequence fragment that encodes the desired HBc chimer prepared as described in Example 1 is ligated into the expression plasmid, which results in the formation of a circular recombinant expression plasmid denominated pSV-Pf.

The expression plasmid pSV-Pf contains an intact *E. coli* ampicillin resistance gene. *E. coli* RR101 (Bethesda Research Laboratories, Gaithersburg, MD), when transformed with pSV-Pf, can thus be selected on the basis of ampicillin resistance for those bacteria containing the plasmid. Plasmid-containing bacteria are then cloned and the clones are subsequently screened for the proper orientation

of the inserted coding gene into the expression
vector.

The above obtained plasmid, pSV-Pf,
containing the gene that encodes a desired HBc chimer
is propagated by culturing *E. coli* containing the
plasmid. The plasmid DNA is isolated from *E. coli*
cultures as described in Sambrook et al., above.

Expression of a chimer is accomplished by
the introduction of pSV-Pf into the mammalian cell
line, e.g., CHO cells, using the calcium phosphate-
mediated transfection method of Graham et al.(1973)
Virol., **52**:456, or a similar technique.

To help ensure maximal efficiency in the
introduction of pSV-Pf into CHO cells in culture, the
transfection is carried out in the presence of a
second plasmid, pSV2NEO (ATCC #37149) and the
cytotoxic drug G418 (GIBCO Laboratories, Grand
Island, N.Y.) as described by Southern et al. (1982)
J. Mol. Appl. Genet., **1**:327. Those CHO cells that
are resistant to G418 are cultured, have acquired
both plasmids, pSV2NEO and pSV-Pf, and are designated
CHO/pSV-Pf cells. By virtue of the genetic
architecture of the pSV-Pf expression vector, a
chimer is expressed in the resulting CHO/pSV-Pf cells
and can be detected in and purified from the
cytoplasm of these cells. The resulting composition
containing cellular protein is separated on a column
as discussed elsewhere herein.

The choice of which expression vector and
ultimately to which promoter a chimer-encoding gene
is operatively linked depends directly on the
functional properties desired, e.g. the location and
timing of protein expression, and the host cell to be
transformed. These are well known limitations

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inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention can direct the replication, and preferably also the expression (for an expression vector) of the chimer gene included in the DNA segment to which it is operatively linked.

In one preferred embodiment, the host that expresses the chimer is the prokaryote, *E. coli*, and a preferred vector includes a prokaryotic replicon; i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell transformed therewith. Such replicons are well known in the art.

Those vectors that include a prokaryotic replicon can also include a prokaryotic promoter region capable of directing the expression of a contemplated HBc chimer gene in a host cell, such as *E. coli*, transformed therewith. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing one or more convenient restriction sites for insertion of a contemplated DNA segment. Typical of such vector plasmids are pUC8, pUC9, and pBR329 available from Biorad Laboratories, (Richmond, CA) and pPL and pKK223-3 available from Pharmacia, Piscataway, NJ.

Typical vectors useful for expression of genes in cells from higher plants and mammals are well known in the art and include plant vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers et al. (1987) *Meth. in Enzymol.*, **153**:253-277 and mammalian expression vectors pKSV-10, above, and pCI-neo (Promega Corp., #E1841, Madison, WI). However,

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several other expression vector systems are known to function in plants including pCaMVCN transfer control vector described by Fromm et al. (1985) *Proc. Natl. Acad. Sci. USA*, **82**:58-24. Plasmid pCaMVCN (available from Pharmacia, Piscataway, NJ) includes the cauliflower mosaic virus CaMV 35S promoter.

The above plant expression systems typically provide systemic or constitutive expression of an inserted transgene. Systemic expression can be useful where most or all of a plant is used as the source to a contemplated chimer molecule or resultant particles or where a large part of the plant is used to provide an oral vaccine. However, it can be more efficacious to express a chimer molecule or particles in a plant storage organ such as a root, seed or fruit from which the particles can be more readily isolated or ingested.

One manner of achieving storage organ expression is to use a promoter that expresses its controlled gene in one or more preselected or predetermined non-photosynthetic plant organs. Expression in one or more preselected storage organs with little or no expression in other organs such as roots, seed or fruit versus leaves or stems is referred to herein as enhanced or preferential expression. An exemplary promoter that directs expression in one or more preselected organs as compared to another organ at a ratio of at least 5:1 is defined herein as an organ-enhanced promoter. Expression in substantially only one storage organ and substantially no expression in other storage organs is referred to as organ-specific expression; i.e., a ratio of expression products in a storage organ relative to another of about 100:1 or greater

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indicates organ specificity. Storage organ-specific
promoters are thus members of the class of storage
organ-enhanced promoters.

Exemplary plant storage organs include the
roots of carrots, taro or manioc, potato tubers, and
the meat of fruit such as red guava, passion fruit,
mango, papaya, tomato, avocado, cherry, tangerine,
mandarin, palm, melons such cantaloupe and
watermelons and other fleshy fruits such as squash,
cucumbers, mangos, apricots, peaches, as well as the
seeds of maize (corn), soybeans, rice, oil seed rape
and the like.

The CaMV 35S promoter is normally deemed to
be a constitutive promoter. However, recent research
has shown that a 21-bp region of the CaMV 35S
promoter, when operatively linked into another,
heterologous usual green tissue promoter, the rbcS-3A
promoter, can cause the resulting chimeric promoter
to become a root-enhanced promoter. That 21-bp
sequence is disclosed in U.S. Patent No. 5,023,179.
The chimeric rbcS-3A promoter containing the 21-bp
insert of U.S. Patent No. 5,023,179 is a useful root-
enhanced promoter herein.

A similar root-enhanced promoter, that
includes the above 21-bp segment is the -90 to +8
region of the CAMV 35S promoter itself. U.S. Patent
No. 5,110,732 discloses that that truncated CaMV 35S
promoter provides enhanced expression in roots and
the radical of seed, a tissue destined to become a
root. That promoter is also useful herein.

Another useful root-enhanced promoter is
the -1616 to -1 promoter of the oil seed rape
(*Brassica napus L.*) gene disclosed in PCT/GB92/00416
(WO 91/13922 published Sep. 19, 1991). *E. coli*

DH5.alpha. harboring plasmid pRlambdaS4 and
bacteriophage lambda.beta.1 that contain this
promoter were deposited at the National Collection of
Industrial and Marine Bacteria, Aberdeen, GB on Mar.
8, 1990 and have accession numbers NCIMB40265 and
NCIMB40266. A useful portion of this promoter can be
obtained as a 1.0 kb fragment by cleavage of the
plasmid with HaeIII.

A preferred root-enhanced promoter is the
mannopine synthase (mas) promoter present in plasmid
pKan2 described by DiRita and Gelvin (1987) *Mol. Gen.
Genet*, **207**:233-241. This promoter is removable from
its plasmid pKan2 as a XbaI-XbalI fragment.

The preferred mannopine synthase root-
enhanced promoter is comprised of the core mannopine
synthase (mas) promoter region up to position -138
and the mannopine synthase activator from -318 to -
213, and is collectively referred to as AmasPmas.
This promoter has been found to increase production
in tobacco roots about 10- to about 100-fold compared
to leaf expression levels.

Another root specific promoter is the about
500 bp 5' flanking sequence accompanying the
hydroxyproline-rich glycopeprotein gene, HRGPnt3,
expressed during lateral root initiation and reported
by Keller et al. (1989) *Genes Dev.*, **3**:1639-1646.
Another preferred root-specific promoter is present
in the about -636 to -1 5' flanking region of the
tobacco root-specific gene ToRBF reported by Yamamoto
et al. (1991) *Plant Cell*, **3**:371-381. The cis-acting
elements regulating expression are more specifically
located by those authors in the region from about
-636 to about -299 5' from the transcription
initiation site. Yamamoto et al. reported steady

state mRNA production from the ToRBF gene in roots, but not in leaves, shoot meristems or stems.

Still another useful storage organ-specific promoter are the 5' and 3' flanking regions of the fruit-ripening gene E8 of the tomato, *Lycopersicon esculentum*. These regions and their cDNA sequences are illustrated and discussed in Deikman et al. (1988) *EMBO J.*, **7**(11):3315-3320 and (1992) *Plant Physiol.*, **100**:2013-2017.

Three regions are located in the 2181 bp of the 5' flanking sequence of the gene and a 522 bp sequence 3' to the poly (A) addition site appeared to control expression of the E8 gene. One region from -2181 to -1088 is required for activation of E8 gene transcription in unripe fruit by ethylene and also contributes to transcription during ripening. Two further regions, -1088 to -863 and -409 to -263, are unable to confer ethylene responsiveness in unripe fruit but are sufficient for E8 gene expression during ripening.

The maize sucrose synthase-1 (Sh) promoter that in corn expresses its controlled enzyme at high levels in endosperm, at much reduced levels in roots and not in green tissues or pollen has been reported to express a chimeric reporter gene, β-glucuronidase (GUS), specifically in tobacco phloem cells that are abundant in stems and roots. Yang et al. (1990) *Proc. Natl. Acad. Sci., U.S.A.*, **87**:4144-4148. This promoter is thus useful for plant organs such as fleshy fruits like melons, e.g. cantaloupe, or seeds that contain endosperm and for roots that have high levels of phloem cells.

Another exemplary tissue-specific promoter is the lectin promoter, which is specific for seed

tissue. The lectin protein in soybean seeds is
encoded by a single gene (Le1) that is only expressed
during seed maturation and accounts for about 2 to
about 5 percent of total seed mRNA. The lectin gene
and seed-specific promoter have been fully
characterized and used to direct seed specific
expression in transgenic tobacco plants. See, e.g.,
Vodkin et al. (1983) *Cell,* **34**:1023 and Lindstrom et
al. (1990) *Developmental Genetics,* **11**:160.

A particularly preferred tuber-specific
expression promoter is the 5' flanking region of the
potato patatin gene. Use of this promoter is
described in Twell et al. (1987) *Plant Mol. Biol.,*
9:365-375. This promoter is present in an about 406
bp fragment of bacteriophage LPOTI. The LPOTI
promoter has regions of over 90 percent homology with
four other patatin promoters and about 95 percent
homology over all 400 bases with patatin promoter
PGT5. Each of these promoters is useful herein. See,
also, Wenzler et al. (1989) *Plant Mol. Biol.,* **12**:41-
50.

Still further organ-enhanced and organ-
specific promoter are disclosed in Benfey et al.
(1988) *Science,* **244**:174-181.

Each of the promoter sequences utilized is
substantially unaffected by the amount of chimer
molecule or particles in the cell. As used herein,
the term "substantially unaffected" means that the
promoter is not responsive to direct feedback control
(inhibition) by the chimer molecules or particles
accumulated in transformed cells or transgenic plant.

Transfection of plant cells using
Agrobacterium tumefaciens is typically best carried
out on dicotyledonous plants. Monocots are usually

most readily transformed by so-called direct gene
transfer of protoplasts. Direct gene transfer is
usually carried out by electroportation, by
polyethyleneglycol-mediated transfer or bombardment
of cells by microprojectiles carrying the needed DNA.
These methods of transfection are well-known in the
art and need not be further discussed herein.
Methods of regenerating whole plants from transfected
cells and protoplasts are also well-known, as are
techniques for obtaining a desired protein from plant
tissues. See, also, U.S. Patents No. 5,618,988 and
5,679,880 and the citations therein.

A transgenic plant formed using
Agrobacterium transformation, electroportation or
other methods typically contains a single gene on one
chromosome. Such transgenic plants can be referred to
as being heterozygous for the added gene. However,
inasmuch as use of the word "heterozygous" usually
implies the presence of a complementary gene at the
same locus of the second chromosome of a pair of
chromosomes, and there is no such gene in a plant
containing one added gene as here, it is believed
that a more accurate name for such a plant is an
independent segregant, because the added, exogenous
chimer molecule-encoding gene segregates
independently during mitosis and meiosis. A
transgenic plant containing an organ-enhanced
promoter driving a single structural gene that
encodes a contemplated HBc chimeric molecule; i.e.,
an independent segregant, is a preferred transgenic
plant.

More preferred is a transgenic plant that
is homozygous for the added structural gene; i.e., a
transgenic plant that contains two added genes, one

gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for enhanced chimer particle accumulation relative to a control (native, non-transgenic) or an independent segregant transgenic plant. A homozygous transgenic plant exhibits enhanced chimer particle accumulation as compared to both a native, non-transgenic plant and an independent segregant transgenic plant.

It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous (heterologous) genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a chimeric HBc molecule. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

A transgenic plant of this invention thus has a heterologous structural gene that encodes a contemplated chimeric HBc molecule. A preferred transgenic plant is an independent segregant for the added heterologous chimeric HBc structural gene and can transmit that gene to its progeny. A more preferred transgenic plant is homozygous for the heterologous gene, and transmits that gene to all of its offspring on sexual mating.

Inasmuch as a gene that encodes a chimeric HBc molecule does not occur naturally in plants, a contemplated transgenic plant accumulates chimeric HBc molecule particles in a greater amount than does

a non-transformed plant of the same type or strain when both plants are grown under the same conditions.

The phrase "same type" or "same strain" is used herein to mean a plant of the same cross as or a clone of the untransformed plant. Where alleic variations among siblings of a cross are small, as with extensively inbred plant, comparisons between siblings can be used or an average arrived at using several siblings. Otherwise, clones are preferred for the comparison.

Seed from a transgenic plant is grown in the field greenhouse, window sill or the like, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for chimeric HBc molecule particle accumulation, preferably in the field, under a range of environmental conditions.

A transgenic plant homozygous for chimeric HBc molecule particle accumulation is crossed with a parent plant having other desired traits. The progeny, which are heterozygous or independently segregatable for chimeric HBc molecule particle accumulation, are backcrossed with one or the other parent to obtain transgenic plants that exhibit chimeric HBc molecule particle accumulation and the other desired traits. The backcrossing of progeny with the parent may have to be repeated more than once to obtain a transgenic plant that possesses a number of desirable traits.

An insect cell system can also be used to express a HBc chimer. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) or baculovirus is used as a vector to

express foreign genes in *Spodoptera frugiperda* cells or in Trichoplusia larvae.

The sequences encoding a chimer can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of chimer sequence renders the polyhedrin gene inactive and produces recombinant virus lacking coat protein. The recombinant viruses can then be used to infect, for example, *S. Frugiperda* cells or *Trichoplusia* larvae in which the HBc chimer can be expressed. E. Engelhard et al. (1994) *Proc. Natl. Acad. Sci., USA*, **91**:3224-3227; and V. Luckow, Insect Cell Expression Technology, pp. 183-218, in Protein Engineering: Principles and Practice, J.L. Cleland et al. eds., Wiley-Liss, Inc, 1996). Heterologous genes placed under the control of the polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) are often expressed at high levels during the late stages of infection.

Recombinant baculoviruses containing the chimeric gene are constructed using the baculovirus shuttle vector system (Luckow et al. (1993) *J. Virol.*, **67**:4566-4579], sold commercially as the Bac-To-Bac™ baculovirus expression system (Life Technologies). Stocks of recombinant viruses are prepared and expression of the recombinant protein is monitored by standard protocols (O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, W.H. Freeman and Company, New York, 1992; and King et al., The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992).

A variety of methods have been developed to operatively link DNA to vectors via complementary

cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Alternatively, synthetic linkers containing one or more restriction endonuclease sites can be used to join the DNA segment to the expression vector, as noted before. The synthetic linkers are attached to blunt-ended DNA segments by incubating the blunt-ended DNA segments with a large excess of synthetic linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase.

Thus, the products of the reaction are DNA segments carrying synthetic linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction endonuclease and ligated into an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the synthetic linker. Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including New England BioLabs, Beverly, MA. A desired DNA segment can also be obtained using PCR technology in which the forward and reverse primers contain desired restriction sites that can be cut after amplification so that the gene can be inserted into the vector. Alternatively PCR products can be directly cloned into vectors containing T-overhangs (Promega Corp., A3600, Madison, WI) as is well known in the art.

The expressed chimeric protein self-assembles into particles within the host cells, whether in single cells or in cells within a multicelled host. The particle-containing cells are harvested using standard procedures, and the cells are lysed using a French pressure cell, lysozyme, sonicator, bead beater or a microfluidizer (Microfluidics International Corp., Newton MA). After clarification of the lysate, particles are precipitated with 45% ammonium sulfate, resuspended in 20 mM sodium phosphate, pH 6.8 and dialyzed against the same buffer. The dialyzed material is clarified by brief centrifugation and the supernatant subjected to gel filtration chromatography using Sepharose® CL-4B. Particle-containing fractions are identified, subjected to hydroxyapatite chromatography, and reprecipitated with ammonium sulfate prior to resuspension, dialysis and sterile filtration and storage at -70°C.

Malarial Inocula and Vaccines

A before-described recombinant HBc chimer. immunogen preferably in particulate form is dissolved or dispersed in an immunogenic effective amount in a pharmaceutically acceptable vehicle composition that is preferably aqueous to form an inoculum or vaccine. When administered to a host animal in need of immunization or in which antibodies are desired to be induced such as a mammal (e.g., a mouse, dog, goat, sheep, horse, bovine, monkey, ape, or human) or bird (e.g., a chicken, turkey, duck or goose), an inoculum induces antibodies that immunoreact with the malarial B cell epitope present in the immunogen. In a vaccine, those induced antibodies also immunoreact *in vivo* with (bind to) the sporozoite and

protect the mammal from malarial infection by the
Plasmodium species whose B cell epitope was present in the
immunogen. A composition that is an inoculum in one
animal can be a vaccine for another where the *Plasmodium*
species against which antibodies are raised does not
infect the animal inoculated, as where an inoculum against
P. falciparum is used to raise antibodies in mice.

The amount of recombinant HBc chimer
immunogen utilized in each immunization is referred
to as an immunogenic effective amount and can vary
widely, depending *inter alia*, upon the recombinant
HBc chimer immunogen, mammal immunized, and the
presence of an adjuvant in the vaccine, as discussed
below. Immunogenic effective amounts for a vaccine
and an inoculum provide the protection or antibody
activity, respectively, discussed hereinbefore.

Vaccines or inocula typically contain a
recombinant HBc chimer immunogen concentration of about 1
microgram to about 1 milligram per inoculation (unit
dose), and preferably about 10 micrograms to about 50
micrograms per unit dose. Immunizations in mice typically
contain 10 or 20 µg of chimer particles.

The term "unit dose" as it pertains to a vaccine
or inoculum of the present invention refers to a
physically discrete unit suitable as an unitary dosage for
animals, each unit containing a predetermined quantity of
active material calculated to individually or collectively
produce the desired immunogenic effect in association with
the required diluent; i.e., carrier, or vehicle. A single
unit dose or a plurality of unit doses can be used to
provide an immunogenic effective amount of recombinant HBc
chimer immunogen.

Vaccines or inocula are typically prepared
from a recovered recombinant HBc chimer immunogen by

dispersing the immunogen in a physiologically tolerable (acceptable) diluent vehicle such as water, saline phosphate-buffered saline (PBS), acetate-buffered saline (ABS), Ringer's solution or the like to form an aqueous composition. The diluent vehicle can also include oleaginous materials such as peanut oil, squalane or squalene as is discussed hereinafter.

The immunogenic active ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, an inoculum or vaccine can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents that enhance the immunogenic effectiveness of the composition.

A contemplated vaccine or inoculum advantageously also includes an adjuvant. Suitable adjuvants for vaccines and inocula of the present invention comprise those adjuvants that are capable of enhancing the antibody responses against B cell epitopes of the chimer, as well as adjuvants capable of enhancing cell mediated responses towards T cell epitopes contained in the chimer. Adjuvants are well known in the art (see, for example, Vaccine Design - The Subunit and Adjuvant Approach, 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X).

Exemplary adjuvants include complete Freund's adjuvant (CFA) that is not used in humans, incomplete Freund's adjuvant (IFA), squalene,

squalane and alum [e.g., Alhydrogel™ (Superfos, Denmark)], which are materials well known in the art, and are available commercially from several sources.

Preferred adjuvants for use with immunogens of the present invention include aluminum or calcium salts (for example hydroxide or phosphate salts). A particularly preferred adjuvant for use herein is an aluminum hydroxide gel such as Alhydrogel™. For aluminum hydroxide gels (alum), the chimer protein is admixed with the adjuvant so that between 50 to 800 micrograms of aluminum are present per dose, and preferably between 400 and 600 micrograms are present.

Another particularly preferred adjuvant for use with an immunogen of the present invention is an emulsion. A contemplated emulsion can be an oil-in-water emulsion or a water-in-oil emulsions. In addition to the immunogenic chimer protein, such emulsions comprise an oil phase of squalene, squalane, peanut oil or the like as are well-known, and a dispersing agent. Non-ionic dispersing agents are preferred and such materials include mono- and di-C_{12}-C_{24}-fatty acid esters of sorbitan and mannide such as sorbitan mono-stearate, sorbitan mono-oleate and mannide mono-oleate. An immunogen-containing emulsion is administered as an emulsion.

Preferably, such emulsions are water-in-oil emulsions that comprise squalene and mannide mono-oleate (Arlacel™ A), optionally with squalane, emulsified with the chimer protein in an aqueous phase. Well-known examples of such emulsions include Montanide™ ISA-720, and Montanide™ ISA 703 (Seppic, Castres, France), each of which is understood to contain both squalene and squalane, with squalene

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predominating in each, but to a lesser extent in Montanide™ ISA 703. Most preferably, Montanide™ ISA-720 is used, and a ratio of oil-to-water of 7:3 (w/w) is used. Other preferred oil-in-water emulsion adjuvants include those disclosed in WO 95/17210 and EP 0 399 843.

The use of small molecule adjuvants is also contemplated herein. One type of small molecule adjuvant useful herein is a 7-substituted-8-oxo- or 8-sulfo-guanosine derivative described in U.S. Patents No. 4,539,205, No. 4,643,992, No. 5,011,828 and No. 5,093,318, whose disclosures are incorporated by reference. Of these materials, 7-allyl-8-oxoguanosine (loxoribine) is particularly preferred. That molecule has been shown to be particularly effective in inducing an antigen-(immunogen-)specific response.

Still further useful adjuvants include monophosphoryl lipid A (MPL) available from Corixa Corp. (see, U.S. Patent No. 4,987,237), CPG available from Coley Pharmaceutical Group, QS21 available from Aquila Biopharmaceuticals, Inc., SBAS2 available from SKB, the so-called muramyl dipeptide analogues described in U.S. Patent No. 4,767,842, and MF59 available from Chiron Corp. (see, U.S. Patents No. 5,709,879 and No. 6,086,901).

More particularly, immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree *Quillaja Saponaria Molina* (e.g. Quil™ A) are also useful. Derivatives of Quil™ A, for example QS21 (an HPLC purified fraction derivative of Quil™ A), and the method of its production is disclosed in U.S. Patent

No.5,057,540. In addition to QS21 (known as QA21),
other fractions such as QA17 are also disclosed.

3-De-O-acylated monophosphoryl lipid A is a
well-known adjuvant manufactured by Ribi Immunochem,
Hamilton, Montana. The adjuvant contains three
components extracted from bacteria, monophosphoryl
lipid (MPL) A, trehalose dimycolate (TDM) and cell
wall skeleton (CWS) (MPL+TDM+CWS) in a 2%
squalene/Tween® 80 emulsion. This adjuvant can be
prepared by the methods taught in GB 2122204B. A
preferred form of 3-de-O-acylated monophosphoryl
lipid A is in the form of an emulsion having a small
particle size less than 0.2 μm in diameter (EP 0 689
454 B1).

The muramyl dipeptide adjuvants include N-
acetyl-muramyl-L-threonyl-D-isoglutamine(thur-MDP),
N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP
11637, referred to as nor-MDP), and N-acetylmuramyl-
L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-
dipalmityol-sn-glycero-3-hydroxyphosphoryloxy)-
ethylamin (CGP) 1983A, referred to as MTP-PE).

Preferred adjuvant mixtures include
combinations of 3D-MPL and QS21 (EP 0 671 948 B1),
oil-in-water emulsions comprising 3D-MPL and QS21 (WO
95/17210, PCT/EP98/05714), 3D-MPL formulated with
other carriers (EP 0 689 454 B1), QS21 formulated in
cholesterol-containing liposomes (WO 96/33739), or
immunostimulatory oligonucleotides (WO 96/02555).
Alternative adjuvants include those described in WO
99/52549 and non-particulate suspensions of
polyoxyethylene ether (UK Patent Application No.
9807805.8).

Adjuvants are utilized in an adjuvant
amount, which can vary with the adjuvant, mammal and

recombinant HBc chimer immunogen. Typical amounts
can vary from about 1 µg to about 1 mg per
immunization. Those skilled in the art know that
appropriate concentrations or amounts can be readily
determined.

Inocula and vaccines are conventionally
administered parenterally, by injection, for example,
either subcutaneously or intramuscularly. Additional
formulations that are suitable for other modes of
administration include suppositories and, in some
cases, oral formulation or by nasal spray. For
suppositories, traditional binders and carriers can
include, for example, polyalkalene glycols or
triglycerides; such suppositories may be formed from
mixtures containing the active ingredient in the
range of 0.5% to 10%, preferably 1-2%. Oral
formulations include such normally employed
excipients as, for example, pharmaceutical grades of
mannitol, lactose, starch, magnesium stearate, sodium
saccharine, cellulose, magnesium carbonate and the
like.

An inoculum or vaccine composition takes
the form of a solution, suspension, tablet, pill,
capsule, sustained release formulation or powder, and
contains an immunogenic effective amount of HBc
chimer, preferably as particles, as active
ingredient. In a typical composition, an immunogenic
effective amount of preferred HBc chimer particles is
about 1 µg to about 1 mg of active ingredient per
dose, and more preferably about 5 µg to about 50 µg
per dose, as noted before.

A vaccine or inoculum is typically
formulated for parenteral administration. Exemplary
immunizations are carried out sub-cutaneously (SC)

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intra-muscularly (IM), intravenusly (IV), intraperitoneally (IP) or intra-dermally (ID).

The HBc chimer particles and HBc chimer particle conjugates can be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein or hapten) and are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived form inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The inocula or vaccines are administered in a manner compatible with the dosage formulation, and in such amount as are therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in intervals (weeks or months) by a subsequent injection or other administration.

Once immunized, the mammal is maintained for a period of time sufficient for the recombinant HBc chimer immunogen to induce the production of a sufficient titer of antibodies that bind to sporozoite. The maintenance time for the production of anti-sporozoite antibodies typically lasts for a period of about three to about twelve weeks, and can include a booster, second immunizing administration of the vaccine. A third immunization is also contemplated, if desired, at a time 24 weeks to five years after the first immunization. It is particularly contemplated that once a protective level titer of anti-sporozoite antibodies is attained, that the vaccinated mammal is preferably maintained at or near that antibody titer by periodic booster immunizations administered at intervals of about 1 to about 5 years.

The production of anti-sporozoite antibodies is readily ascertained by obtaining a plasma or serum sample from the immunized mammal and assaying the antibodies therein for their ability to bind to a synthetic circumsporozoite immunodominant antigen [e.g. the *P. falciparum* CS protein peptide (NANP)$_5$ used herein] in an ELISA assay as described hereinafter or by another immunoassay such as a Western blot as is well known in the art. Most preferable is the use of the indirect immunofluorescence assay (IFA), in which intact sporozoites are employed as the capture antigen, discussed hereinafter.

It is noted that the before-described anti-CS antibodies so induced can be isolated from the blood of the host mammal using well known techniques, and then reconstituted into a second vaccine for

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passive immunization as is also well known. Similar techniques are used for gamma-globulin immunizations of humans. For example, antiserum from one or a number of immunized hosts can be precipitated in aqueous ammonium sulfate (typically at 40-50 percent of saturation), and the precipitated antibodies purified chromatographically as by use of affinity chromatography in which (NANP)$_5$ is utilized as the antigen immobilized on the chromatographic column.

Inocula are preparations that are substantially identical to vaccines, but are used in a host mammal in which antibodies to malaria are desired to be induced, but in which protection from malaria is not desired. In one example, a vaccine against *P. falciparum* of *P. vivax* can be used in mice as an inoculum to induce antibody production and not be a vaccine because neither malarial species can infect mice. Similarly, a similar inoculum can be used in a horse or sheep to induce antibody production against either or both malarial species for use in a passive immunization in yet another animal such as humans.

Best Mode for Carrying Out the Invention

Without further elaboration, it is believed that one skilled in the art can, using the preceding description and the detailed examples below, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limiting of the remainder of the disclosure in any way whatsoever.

Example 1: B Cell Epitope-Containing

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<u>Chimer Preparation</u>

A.   Preparation of plasmid vector pKK223-
     3N, a modified form of pKK223-3

Plasmid vector pKK223-3 (Pharmacia) was
modified by the establishment of a unique NcoI
restriction site to enable insertion of HBc genes as
NcoI-HindIII restriction fragments and subsequent
expression in *E.coli* host cells. To modify the
pKK223-3 plasmid vector, a new SphI-HindIII fragment
was prepared using the PCR primers pKK223-3/433-452-F
and pKK223-NcoI-mod-R, and pKK223-3 as the template.

     This PCR fragment was cut with the
restriction enzymes SphI and HindIII to provide a 467
bp fragment that was then ligated with a 4106 bp
fragment of the pKK223-3 vector, to effectively
replace the original 480 bp SphI-HindIII fragment.
The resultant plasmid (pKK223-3N) is therefore 13 bp
shorter than the parent plasmid and contains modified
nucleotide sequence upstream of the introduced NcoI
site (see Fig. 1 in which the dashes indicate the
absent bases). The final plasmid, pKK223-3N, has a
size of 4573 bp. Restriction sites in plasmid
pKK223-3N are indicated in Fig. 1, and the nucleotide
changes made to pKK223-3 to form plasmid pKK223-3N
are indicated by an underline as shown below.

pKK223-3/433-452-F   GGTGCATGCAAGGAGATG   SEQ ID NO:27

pKK223-NcoI-mod-R
GCGAAGCTTCGGATC<u>ccatgg</u>TTTTTTCCTCCTTATGTGAAATTGTTATCCG-
          CTC   SEQ ID NO:28

     B.   Preparation of V1
          and V2 Cloning Vectors

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Modified HBc149 genes, able to accept the directional insertion of synthetic dsDNA fragments into the immunodominant loop region, were constructed using PCR. (The plasmid accepting inserts between amino acids E77 and D78 was named V1, whereas the plasmid accepting inserts between D78 and P79 was named V2.) The HBc149 gene was amplified in two halves using two PCR primer pairs, one of which amplifies the amino terminus, the other amplifies the carboxyl terminus. For V1, the products of the PCR reactions (N- and C-terminus) are both 246 bp fragments; for V2, the products are a 249 bp (N-terminus) and a 243 bp fragment (C-terminus).

The N-terminal fragments prepared were digested with NcoI and EcoRI, and the C-terminal fragments were digested with EcoRI and HindIII. The V1 and V2 fragments pairs were then ligated together at the common EcoRI overhangs. The resultant NcoI-HindIII fragments were then ligated into the pKK223-3N vector, which had been prepared by digestion with NcoI and HindIII.

To insert B cell epitopes into the V1 and V2 plasmids, the plasmids were digested with EcoRI and SacI restriction enzymes. Synthetic dsDNA fragments containing 5' EcoRI and 3' SacI overhangs were then inserted. In both cases, V1 and V2, glycine-isoleucine (EcoRI) and glutamic acid-leucine (SacI) amino acid pairs, coded for by the restriction sites, flank the inserted B cell epitopes. The inserted restriction sites are underlined in the primers below.

V1

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA   SEQ ID NO:29

HBc-E77/EcoRI-R

5'-GCGGAATTCCTTCCAAATTAACACCCACC   SEQ ID NO:30

HBc-D78/EcoRI-SacI-F

5'-CGCGAATTCAAAAAGAGCTCGATCCAGCGTCTAGAGAC

                                               SEQ ID NO:31

HBc149/HindIII-R

5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG   SEQ ID NO:32

        V2

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA   SEQ ID NO:29

HBc-D78/EcoRI-R

5'-GCGGAATTCCATCTTCCAAATTAACACCCAC   SEQ ID NO:186

HBc-P79/EcoRI-SacI-F

5'-CGCGAATTCAAAAAGAGCTCCCAGCGTCTAGAGACCTAG

                                               SEQ ID NO:34

HBc149/HindIII-R

5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG   SEQ ID NO:32

        C.   Preparation of V7 Cloning Vector

        To enable the fusion of T cell epitopes to
the C terminus of a HBc chimer, a new vector, V7, was
constructed.   Unique EcoRI and SacI restriction sites

were inserted between valine-149 and the HindIII site
to facilitate directional insertion of synthetic
dsDNAs into EcoRI-HindIII (or EcoRI-SacI) restriction
sites. The pair of PCR primers below was used to
amplify the HBc 149 gene with a NcoI restriction site
at the amino-terminus and EcoRI, SacI and HindIII
sites at the carboxyl-terminus. The product of the
PCR reaction (479 bp) was digested with NcoI/HindIII
and cloned into pKK223-3N to form V7.

　　　　To insert T cell epitopes, the plasmid (V7)
was digested EcoRI/HindIII (or EcoRI-SacI) and
synthetic dsDNA fragments having EcoRI/HindIII (or
EcoRI/SacI) overhangs, were ligated into V7. For all
V7 constructs, the final amino acid of native HBc
(valine-149) and the first amino acid of the inserted
T cell epitope are separated by a glycine-isoleucine
dipeptide sequence coded for by the nucleotides that
form the EcoRI restriction site. For epitopes
inserted at EcoRI/SacI, there are additional glutamic
acid-leucine residues after the T cell epitope, prior
to the termination codon, contributed by the SacI
site. Restriction sites are again underlined in the
primers shown.

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA　　　　　　SEQ ID NO:29

HBc149/SacI-EcoRI-H3-R

5'-CGCAAGCTTAGAGCTCTTGAATTCCAACAACAGTAGTCTCCG

　　　　　　　　　　　　　　　　　　　SEQ ID NO:33

　　　　D.　Preparation of V12
　　　　　　Expression Constructs

V12 vectors, which contain B cell epitopes
between amino acids 78 and 79, as well as T cell
epitopes downstream of valine-149, were constructed
from V2 and V7 vectors. The carboxyl terminus of a
V7 vector containing a T cell epitope inserted at
EcoRI/HindIII was amplified using two PCR primers
(HBc-P79/SacI-F and pKK223-2/4515-32R) to provide a
dsDNA fragment corresponding to amino acids 79-149
plus the T cell epitope, flanked with SacI and
HindIII restriction sites.

The PCR products were cut with SacI and
HindIII and then cloned into the desired V2 vector
prepared by cutting with the same two enzymes. The
PCR primers shown are amenable for the amplification
of the carboxyl terminus of all V7 genes,
irrespective of the T cell epitope present after
amino acid 149 of the HBc gene.

One exception to the generality of this
approach was in the preparation of the V12 constructs
containing the Pf-CS(C17A) mutation, which were
prepared from existing V12 constructs. In this case,
V12 constructs were amplified with HBc149/NcoI-F (SEQ
ID NO:29) and the mis-match reverse PCR primer (SEQ
ID NO: 104), which facilitated the C17A mutation.
The resultant PCR product was digested with NcoI and
HindIII and cloned back into pKK223-3N (previously
cut with the same enzymes). Restriction sites are
underlined.

HBc-P79/SacI-F   5'-CGC<u>GAGCTC</u>CCAGCGTCTAGAGACCTAG

                                                    SEQ ID NO:35

pKK223-2/4515-32R   5'-GTATCAGGCTGAAAATC

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E. *P.falciparum* CS-repeat B cell
    Epitopes Inserted into V2

For V2 and V7 constructs, synthetic dsDNA
fragments coding for the B (V2) or T cell epitope
(V7) of interest were inserted into EcoRI/SacI
restriction sites. Synthetic dsDNA fragments,
encoding B and T cell epitopes of interest, were
prepared by mixing complementary single stranded DNA
oligonucleotides at equimolar concentrations, heating
to 95°C for 5 minutes, and then cooling to room
temperature at a rate of -1 °C per minute. This
annealing reaction was performed in TE buffer. The
double-stranded DNAs are shown below with the encoded
epitope sequence shown above. The pound symbol, #,
is used in some of the amino acid residue sequences
that follow to indicate the presence of a stop codon.

Pf1

```
   I  N  A  N  P  N  A  N  P  N  A  N  P  N  A
AATTAACGCTAATCCGAACGCTAATCCGAACGCTAATCCGAACGCTA
    TTGCGATTAGGCTTGCGATTAGGCTTGCGATTAGGCTTGCGAT
```

```
         N   P   E   L            SEQ ID NO: 37
         ATCCGGAGCT               SEQ ID NO: 38
         TAGGCC                   SEQ ID NO: 39
```

Pf3

  I N A N P N V D P N A N P N A N P
AATTAACGCTAATCCGAACGTTGACCCGAACGCTAATCCGAACGCTAATCCGA
    TTGCGATTAGGCTTGCAACTGGGCTTGCGATTAGGCTTGCGATTAGGCT

N A N P N V D P N A N P E L SEQ ID NO:40
ACGCTAATCCGAACGTTGACCCGAACGCTAATCCGGAGCT SEQ ID NO:41
TGCGATTAGGCTTGCAACTGGGCTTGCGATTAGGCCTCGAGG

SEQ ID NO:42

Pf3.1

  I N A N P N V D P N A N P N A N P
AATTAACGCGAATCCGAACGTGGATCCGAATGCCAACCCTAACGCCAACCC
TTGCGCTTAGGCTTGCACCTAGGCTTACGGTTGGGATTGCGGTTGGG

      N A N P E L   SEQ ID NO:43
    AAATGCGAACCCAGAGCT   SEQ ID NO:44
    TTTACGCTTGGGTC   SEQ ID NO:45

Pf3.2

  I N A N P N A N P N A N P N V D P
AATTAACGCGAATCCGAATGCCAACCCTAACGCCAACCCAAACGTGGATCCGA
    TTGCGCTTAGGCTTACGGTTGGGATTGCGGTTGGGTTTGCACCTAGGCT

      N A N P E L   SEQ ID NO:46
    ATGCGAACCCAGAGCT   SEQ ID NO:47
    TACGCTTGGGTC   SEQ ID NO:48

Pf3.3

```
  I   N   A   N   P   N   V   D   P   N   A   N   P   N   A   N   P
AATTAACGCGAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAA
    TTGCGCTTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTT
```

```
N   A   N   P   N   V   D   P   N   A   N   P   E   L   SEQ ID NO:49
ACGCCAACCCGAATGTTGACCCCAATGCCAATCCGGAGCT   SEQ ID NO:50
TGCGGTTGGGCTTACAACTGGGGTTACGGTTAGGCC       SEQ ID NO:51
```

Pf3.4

```
  I   N   P   N   V   D   P   N   A   N   P   N   A   N   P   N   A
AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
    TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT
```

```
        N   P   N   V   E   L            SEQ ID NO:52
        ACCCGAATGTTGAGCT                 SEQ ID NO:53
        TGGGCTTACAAC                     SEQ ID NO:54
```

Pf3.5

```
  I   N   P   N   V   D   P   N   A   N   P   N   A   N   P   N   A
AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
    TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT
```

```
        N   P   N   V   D   P   E   L        SEQ ID NO:55
        ACCCGAATGTTGACCCTGAGCT               SEQ ID NO:56
        TGGGCTTACAACTGGGAC                   SEQ ID NO:57
```

Pf3.6

```
      I  N  P  N  V  D  P  N  A  N  P  N  A  N  P  N  A
    AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
       TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT


           N  P  N  V  D  P  N  A  E  L     SEQ ID NO:58
         ACCCGAATGTTGACCCTAATGCTGAGCT         SEQ ID NO:59
         TGGGCTTACAACTGGGATTACGAC             SEQ ID NO:60
```

Pf3.7

```
      I  N  V  D  P  N  A  N  P  N  A  N  P  N  A  N  P
    AATTAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGA
       TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT


           N  V  E  L              SEQ ID NO:61
         ATGTTGAGCT                 SEQ ID NO:62
         TACAAC                     SEQ ID NO:63
```

Pf3.8

```
      I  N  V  D  P  N  A  N  P  N  A  N  P  N  A  N  P
    AATTAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGA
       TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT


           N  V  D  P  E  L             SEQ ID NO:64
         ATGTTGACCCTGAGCT                SEQ ID NO:65
         TACAACTGGGAC                    SEQ ID NO:66
```

Pf3.9

```
    I   N   V   D   P   N   A   N   P   N   A   N   P   N   A   N   P
AATTAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGA
    TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT
```

```
        N   V   D   P   N   A   E   L           SEQ ID NO:67
        ATGTTGACCCTAATGCTGAGCT                  SEQ ID NO:68
        TACAACTGGGATTACGAC                      SEQ ID NO:69
```

Pf3.10

```
    I   D   P   N   A   N   P   N   A   N   P   N   A   N   P
AATTGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACC
    CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGG
```

```
        N   V   E   L           SEQ ID NO:70
        CGAATGTTGAGCT           SEQ ID NO:71
        GCTTACAAC              SEQ ID NO:72
```

Pf3.11

```
    I   D   P   N   A   N   P   N   A   N   P   N   A   N   P   N   V
AATTGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGAATGTTG
    CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCTTACAAC
```

```
        D   P   E   L           SEQ ID NO:73
        ACCCTGAGCT             SEQ ID NO:74
        TGGGAC                SEQ ID NO:75
```

Pf3.12

```
      I   D   P   N   A   N   P   N   A   N   P   N   A   N   P   N   V
   AATTGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGAATGTTG
         CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCTTACAAC
```

```
            D   P   N   A   E   L              SEQ ID NO:76
         ACCCTAATGCCGAGCT                      SEQ ID NO:77
         TGGGATTACGGC                          SEQ ID NO:78
```

F. *P.falciparum* universal T cell epitope

Pf-UTC (PF/CS326-345)

```
      I   E   Y   L   N   K   I   Q   N   S   L   S   T   E   W   S   P
   AATTGAATATCTGAACAAAATCCAGAACTCTCTGTCCACCGAATGGTCTCCGT
         CTTATAGACTTGTTTTAGGTCTTGAGAGACAGGTGGCTTACCAGAGGCA
```

```
            C   S   V   T   #   #              SEQ ID NO:79
         GCTCCGTTACCTAGTA                      SEQ ID NO:80
         CGAGGCAATGGATCATTCGA                  SEQ ID NO:81
```

P.vivax CS-repeat B cell epitopes

Pv-T1A

```
      I   P   A   G   D   R   A   D   G   Q   P   A   G   D   R   A   A
   AATTCCGGCTGGTGACCGTGCAGATGGCCAGCCAGCGGGTGACCGCGCTGCAG
         GGCCGACCACTGGCACGTCTACCGGTCGGTCGCCCACTGGCGCGACGTC
```

```
            G   Q   P   A   G   E   L          SEQ ID NO:82
         GCCAGCCGGCTGGCGAGCT                   SEQ ID NO:83
         CGGTCGGCCGACCGC                       SEQ ID NO:84
```

Pv-T1B

```
      I   D   R   A   A   G   Q   P   A   G   D   R   A   D   G   Q   P
AATTGACAGAGCAGCCGGACAACCAGCAGGCGATCGAGCAGACGGACAGCCCG
      CTGTCTCGTCGGCCTGTTGGTCGTCCGCTAGCTCGTCTGCCTGTCGGGC
```

A G E L	SEQ ID NO:85
CAGGGGAGCT	SEQ ID NO:86
GTCCCC	SEQ ID NO:87

Pv-T2A

```
      I   A   N   G   A   G   N   Q   P   G   A   N   G   A   G   D   Q
AATTGCGAACGGCGCCGGTAATCAGCCGGGGGGCAAACGGCGCGGGTGATCAAC
      CGCTTGCCGCGGCCATTAGTCGGCCCCCGTTTGCCGCGCCCACTAGTTG
```

P G E L	SEQ ID NO:88
CAGGGGAGCT	SEQ ID NO:89
GTCCCC	SEQ ID NO:90

Pv-T2B

```
      I   A   N   G   A   D   N   Q   P   G   A   N   G   A   D   D   Q
AATTGCGAACGGCGCCGATAATCAGCCGGGTGCAAACGGGGCGGATGACCAAC
      CGCTTGCCGCGGCTATTAGTCGGCCCACGTTTGCCCCGCCTACTGGTTG
```

P G E L	SEQ ID NO:91
CAGGCGAGCT	SEQ ID NO:92
GTCCGC	SEQ ID NO:93

Pv-T2C
```

```
    I  A  N  G  A  G  N  Q  P  G  A  N  G  A  G  D  Q
AATTGCGAACGGCGCCGGTAATCAGCCGGGAGCAAACGGCGCGGGGGATCAAC
    CGCTTGCCGCGGCCATTAGTCGGCCCTCGTTTGCCGCGCCCCCTAGTTG


    P  G  A  N  G  A  D  N  Q  P  G  A  N  G  A  D  D
CAGGCGCCAATGGTGCAGACAACCAGCCTGGGGCGAATGGAGCCGATGACC
GTCCGCGGTTACCACGTCTGTTGGTCGGACCCCGCTTACCTCGGCTACTGG


        Q   P   G   E   L                 SEQ ID NO:94
        AACCCGGCGAGCT                     SEQ ID NO:95
        TTGGGCCGC                         SEQ ID NO:96


PV-T3


    I   E   P   G   A   N   Q   E   G   G   A   A   A   P   G   A   N
AATTGCGCCGGGCGCCAACCAGGAAGGTGGGGCTGCAGCGCCAGGAGCCAATC
    CGCGGCCCGCGGTTGGTCCTTCCACCCCGACGTCGCGGTCCTCGGTTAG


        Q   E   G   G   A   A   E   L     SEQ ID NO:97
        AAGAAGGCGGTGCAGCGGAGCT            SEQ ID NO:98
        TTCTTCCGCCACGTCGCC                SEQ ID NO:99


Example 2:   *P.vivax* universal T cell epitope


Pv-UTC


    I   E   Y   L   D   K   V   R   A   T   V   G   T   E   W   T   P
AATTGAATATCTGGATAAAGTGCGTGCGACCGTTGGCACGGAATGGACTCCGT
    CTTATAGACCTATTTCACGCACGCTGGCAACCGTGCCTTACCTGAGGCA


        C   S   V   T   #   #             SEQ ID NO:100
        GCAGCGTGACCTAATA                  SEQ ID NO:101
        CGTCGCACTGGATTATTCGA              SEQ ID NO:102
```

A. PCR primers for site-directed
    mutagenesis

Pf-CS(C17A)-R   SEQ ID NO:103

        # # T V S A P S W E T S
GCCAAGCTTACTAGGTAACGGAGGCCGGAGACCATTCGGTGG
    HindIII   SEQ ID NO:104

        B. PCR Primers for Truncation and
            Cysteine Addition at C-terminus
        To modify the C-terminus of HBc chimer
genes, either via the addition of cysteine residues
or varying the length of the HBc gene, PCR reactions
were performed using HBc149 as template with the
HBc/NcoI-F primer and a reverse primer (e.g.
HBc149+C/HindIII-R) that directed the desired
modification of the C-terminus. PCR products were
digested with NcoI and HindIII (whose restriction
sites are underlined), and cloned into pKK223-3N at
the same restriction sites.

HBc149/NcoI-F   SEQ ID NO:105
            M D I D P Y
5'-TTGGGCCATGGACATCGACCCTTA   SEQ ID NO:29

HBc149+C/HindIII-R   SEQ ID NO:106
        # # C V V T T E P L
5'-CGCAAGCTTACTAGCAAACAACAGTAGTCTCCGGAAG
                                     SEQ ID NO:107

HBc144/HindIII-R   SEQ ID NO:108

     # P   L   T   S   L   I   P

CGCAAGCTTACGGAAGTGTTGATAGGATAGGG   SEQ ID NO:109

HBc142/HindIII-R   SEQ ID NO:110

     # T   S   L   I   P   A   N   P

CGCAAGCTTATGTTGATAGGATAGGGGCATTTGG   SEQ ID NO:111

HBc140/HindIII-R   SEQ ID NO:112

     # L   I   P   A   N   P   P

CGCAAGCTTATAGGATAGGGGCATTTGGTGG   SEQ ID NO:113

HBc139/HindIII-R   SEQ ID NO:114

     # I   P   A   N   P   P

GCGAAGCTTAGATAGGGGCATTTGGTGG   SEQ ID NO:115

HBc138/HindIII-R   SEQ ID NO:116

     # P   A   N   P   P   R

CGCAAGCTTAAGGGGCATTTGGTGGTCT   SEQ ID NO:117

HBc138+C/HindIII-R   SEQ ID NO:118

     # C   P   A   N   P   P   R

GCGAAGCTTAGCAAGGGGCATTTGGTGGTCT   SEQ ID NO:119

HBc137/HindIII-R   SEQ ID NO:120

     # A   N   P   P   R   Y   A

GCGAAGCTTAGGCATTTGGTGGTCTATAGC   SEQ ID NO:121

HBc137+C/HindIII-R   SEQ ID NO:122

    # C ·A   N   P   P ·R Y   A

GCGAAGCTTAGCAGGCATTTGGTGGTCTATAA   SEQ ID NO:123

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HBc136/HindIII-R   SEQ ID NO:124
        #   N   P   P   R   Y   A   P
CGCAAGCTTAATTTGGTGGTCTATAAGCTGG   SEQ ID NO:125

Example 3: Assay Procedures

        A. Antigenicity
        1. Particle ELISA
        Purified particles were diluted to a
concentration of 10 μg/mL in coating buffer (50 mM
sodium bicarbonate, pH 9.6) and coated onto the wells
of ELISA strips (50 μL/well). The ELISA strips were
incubated at room temperature overnight (about 18
hours). Next morning, the wells were washed with
ELISA wash buffer [phosphate buffered saline (PBS),
pH 7.4, 0.05% Tween®-20] and blocked with 3% BSA in
PBS for 1 hour (75 μL/well). ELISA strips were
stored, dry, at -20°C until needed.

        To determine the antigenicity of particles,
antisera were diluted using 1% BSA in PBS and 50
μL/well added to antigen-coated ELISA wells. Sera
were incubated for 1 hour, washed with ELISA wash
buffer (above) and probed using an anti-mouse (IgG)-
HRP (The Binding Site, San Diego, CA; HRP =
horseradish peroxidase) conjugate (50 μL/well) or
other appropriate antibody for 30 minutes. After
washing with ELISA wash buffer the reaction was
visualized by the addition of TM blue substrate (50
μL/well). After 10 minutes, the reaction was stopped
by the addition of 1N H_2SO_4 (100 μL/well) and read on
an ELISA plate reader set at 450 nm.

2. Synthetic Peptide ELISA

A 20 amino acid residue synthetic peptide
(NANP)$_5$ was diluted to a concentration of 2 μg/mL in
coating buffer (50 mM sodium bicarbonate, pH 9.6) and
coated onto the wells of ELISA strips (50 μL/well).
Peptides were dried onto the wells by incubating
overnight (about 18 hours), in a hood with the
exhaust on. Next morning, the wells were washed with
ELISA wash buffer (phosphate buffered saline, pH 7.4,
0.05% Tween®-20) and blocked with 3% BSA in PBS (75
μL/well) for 1 hour. ELISA strips were stored, dry,
at -20°C until needed.

To determine antibody antigenicity of
particles, antisera (monoclonal or polyclonal) were
diluted using 1% BSA in PBS, and 50 μL/well added to
antigen-coated ELISA wells. Sera were incubated for
1 hour, washed with ELISA wash buffer, and probed
using an anti-mouse(IgG)-HRP conjugate or other
antibody (as above at 50 μL/well) for 30 minutes,
washed again with ELISA wash buffer, and then
visualized by the addition of TM blue substrate (50
μL/well). After 10 minutes, the reaction was stopped
by the addition of 1N H_2SO_4 (100 μL/well) and read on
an ELISA plate reader set at 450 nm.

B. Immunogenicity of Particles

To assay the immunogenicity of particles,
mice were immunized, IP, with 20 μg of particles in
Freund's complete adjuvant, and then boosted at 4
weeks with 10 μg in Freund's incomplete adjuvant.
Mice were bled at 2, 4, 6, and 8 weeks.

C. Sporozoite IFA

Indirect immunofluorescence assay (IFA) was carried out using glutaraldehyde-fixed *P. falciparum* sporozoites and FITC-labeled anti-mouse IgG (gamma-chain specific) (Kirkegaard and Perry, Gaithersburg, MD) to detect bound antibody [Munesinghe *et al.*, *Eur.J.Immunol.* 1991, 21, 3015-3020]. Sporozoites used were dissected from the salivary glands of *Anopheles* mosquitoes infected by feeding on *P.falciparum* (NF54 isolate) gametocytes derived from *in vitro* cultures.

Example 4: Expression of Recombinant
           Chimer HBc Particles

A.   Effect of Insertion
     Position on Immunogenicity

Antibody titers (1/reciprocal dilution) were measured for mice immunized with HBc particles containing the *P. f*-CS B cell epitope (NANP)$_4$, inserted either between amino acids E77/D78 or D78/P79, or by using a loop replacement approach (CS-2) [discussed in Schodel et al., (1994) *J. Exp. Med.*, 180:1037-1046, using complete Freund's adjuvant]. Mice were immunized with a single 20 μg dose, IP, with adjuvant as noted before, and antibody titers determined in an ELISA using immobilized (NANP)$_5$ synthetic peptide. The results of those studies are shown in Table 1, below.

Table 1

Time	CS-2*	E77/D78 (V1)	D78/P79 (V2)
2 weeks	0	2,560	2,560
4 weeks	640	2,560	40,960

*Schodel et al., (1994) *J.Exp.Med.*, 180:1037-1046.

Another comparison was made of insertion position of the NANP CS-repeat epitope on immunogenicity, using BALB/c mice. Antibody titers induced by the CS-2 particle of Schodel et al. were compared to titers achieved using the same $(NANP)_4$ B cell epitope, inserted between HBc positions 78 and 79, and using the above V2.Pf1 particles as immunogen. Sera were analyzed 4 weeks after primary (1°) and 2 weeks after booster (2°) immunization, and the results are shown in Table 2, below.

Table 2

Chimer	Primary	Booster
CS-2	0	640*
V2.Pf1	10,240	655,360

* Schodel et al., (1994) *J.Exp.Med.*, 180:1037-1046

A similar comparison of insertion position of the NANP CS-repeat epitope on immunogenicity was made using B10.S mice, and the results are shown in Table 3.

Table 3

Chimer	Primary	Booster
CS-2	640*	20,480*
V2.Pf1	163,840	655,360

* Schodel et al., (1994) *J.Exp.Med.*, 180:1037-1046

The effect on the immunogenicity of HBc chimer particles (ELISA, F1 mice) that include the minor B cell epitope, NANPNVDP (SEQ ID NO:126) that includes the sequence NVDP (SEQ ID NO: 185), along with a repeated NANP sequence was examined. A HBc chimer was expressed that contained the sequence NANPNVDP(NANP)$_3$NVDP (SEQ ID NO:2; V12.Pf3) inserted between HBc positions 78 and 79. The resulting ELISA data were compared to titers obtained using the tetrameric repeat (NANP)$_4$ B cell epitope (V12.Pf1) or the dimer of the minor B cell epitope at the same position (V12.Pf7). Each of these three chimers contained a Domain IV that included the HBc sequence from position 141 through 149, bonded to the *P. falciparum* universal T cell epitope as the C-terminal sequence. The results of these studies using primary and booster immunizations as discussed before and using adjuvants, are shown below in Table 4.

Table 4

Chimer	Primary	Booster
V12.Pf1	163,840	655,360
V12.Pf3	2,621,440	10,485,760
V12.Pf7	2,560	

The observed greater than 20-fold increase in immunogenicity by including the 'minor' repeat epitope was quite unexpected. Because V12.Pf3 was

not well expressed by *E.coli*, variants of the Pf3
epitope NANPNVDP(NANP)$_3$NVDP (SEQ ID NO:2) were
constructed that had similar antigenicity to Pf3, but
with increased expression levels, as shown below.
Only constructs 3.1 and 3.2 were assayed for
immunogenicity.

   Relative expression levels of recombinant
chimer HBc/*P. falciparum* particles and antigenicities
for monoclonal antibodies specific for the CS
epitopes (NANP)$_4$ and (NANPNVDP) are shown in Table 5
below. Relative expression levels are as follows;
****=75-125 mg/L; ***=50-75 mg/L; **=25-50 mg/L.
Antigenicity was determined by end point titer
dilutions for the monoclonal antibodies [MoAb 2A10
for (NANP)$_4$; MoAb 2B6D8 for NANPNVDP; and *P. vivax*
Rpt. MoAb 2F2 were provided by E. Nardin of New York
University Medical Center]. The data were normalized
such that the lowest titer is expressed as 1. For
example, V12.Pf3 was 165 fold more antigenic than
V12.Pf3.10 for the (NANP)$_4$-specific monoclonal, and
26-fold more antigenic than V12.Pf3.2 for the
NANPNVDP-specific monoclonal antibody. N.D.= no
detectable antibody binding. [Note: V12.Pf3.7 was not
expressed due to a mutation in the expression vector;
it was not examined further because similar
constructs were not antigenic, and re-cloning was
therefore not a worthwhile endeavor.]

Table 5

Name	*P. falciparum* B Cell Epitope	Relative Expression	Antigenicity (NANP)$_4$	NANPNVDP
V12.Pf1	(NANP)$_4$ SEQ ID NO:1	★★★★	33	ND
V12.Pf3	NANPNVDP(NANP)$_3$NVDP SEQ ID NO:2	★★	165	31
V12.Pf3.1	NANPNVDP(NANP)$_3$ SEQ ID NO:3	★★★★	33	31
V12.Pf3.2	(NANP)$_3$NVDPNANP SEQ ID NO:4	★★★	33	1.2
V12.Pf3.3	NANPNVDP(NANP)$_3$- NVDPNANP SEQ ID NO:5	★★	5	1
V12.PF3.4	NPNVDP(NANP)$_3$NV SEQ ID NO:6	★★★★	5	5
V12.PF3.5	NPNVDP(NANP)$_3$NVDP SEQ ID NO:7	★★★★	5	5
V12.PF3.6	NPNVDP(NANP)$_3$- NVDPNA SEQ ID NO:8	★★★★	5	5
V12.PF3.7	NVDP(NANP)$_3$NV SEQ ID NO:9	−	−	−
V12.PF3.8	NVDP(NANP)$_3$NVDP SEQ ID NO:10	★★★★	5	1
V12.PF3.9	NVDP(NANP)$_3$NVDPNA SEQ ID NO:11	★★★	5	ND
V12.PF3.10	DP(NANP)$_3$NV SEQ ID NO:12	★★★★	1	ND
V12.PF3.11	DP(NANP)$_3$NVDP SEQ ID NO:13	★★★★	5	ND
V12.PF3.12	DP(NANP)$_3$NVDPNA SEQ ID NO:14	★★★	5	ND

Immunogenicity of selected HBc chimer particles containing variants of the Pf3 epitope were assayed as described above. Sera were analyzed by ELISA 4 weeks after primary (1°) and 4 weeks after booster (2°) immunizationsd. The data obtained are shown in Table 6, below, in which the "Name" of the chimer and the corresponding sequence of the B cell immunogen are as illustrated above.

Table 6

NAME	PRIMARY	SECONDARY
V12.Pf1	40,960	655,360
V12.Pf3	2,621,440	10,485,760
V12.Pf3.1	2,621,440	10,485,760
V12.Pf3.2	2,621,440	2,621,440

Surprisingly, a version that contained one copy of the NANPNVDP repeat (V12.Pf3.1; SEQ ID NO: 126) was as immunogenic (and expressed better) as a version containing 2 copies (V12.Pf3), despite being 5-fold less antigenic for the NANP monoclonal antibody.

B. Expression failures

Several additional epitopes have been attempted to be placed into the HBc loop (Domain II) between positions 78 and 79 (as in V2.Pf1), and have failed to be expressed for reasons unknown. Table 7,

below, enumerates those epitopes that have failed to express when inserted between D78 and P79 (V2) in a HBc chimer.

Table 7

Designation	Source of Epitope	Epitope (single letter)
V2.FGF-1 (N7-K12)	Human FGF-1	NYKKPK SEQ ID NO:127
V2.FGF-1 (K118-H124)	Human FGF-1	KRGPRTH SEQ ID NO:128
V2.Arom-479	P450 Aromatase	LHPDETKNMLEMIFTPRNSDR SEQ ID NO:129
V2.HIV3.1	HIV-1 (gp120)	RIKQI SEQ ID NO:130
V2.HIV4.1	HIV-1 (gp120)	RIKQIGMPGGK SEQ ID NO:131
V2.HIV5.1	HIV-1 (gp41)	LLELDKWASL SEQ ID NO:132
V2.HIV6.1	HIV-1 (gp41)	EQELLELDKWASLW SEQ ID NO:133
V2.HIV9.1	HIV-1 (gp41)	VQQQNNLLRAIEAQQHLL-QLTVWGIKQLQARIL SEQ ID NO:134
V2.HIV10.1	HIV-1 (gp41)	HLLQLTVWGIKQLQAR SEQ ID NO:135
V2.HIV12.1	HIV-1 (gp41)	YTHIIYSLIEQSQNQQEK-NEQELLALDKWASLWNWF SEQ ID NO:136
V2.HIV13.1	HIV-1 (gp41)	YTHIIYSLIEQSQN-QQEKNEQELLEL SEQ ID NO:137
V2.1A2(351-370)	Human P450-1A2	GRERRPRLSDRPQLPYLEA SEQ ID NO:138
V2.2D6(129-148)	Human P450-2D6	REQRRFSVSTLRNLGLGKKS SEQ ID NO:139
V2.Py-B1	P. yoelii (TRAP)	PNKLPRSTAVVHQLKRKH SEQ ID NO:140
V2.Py-B3	P. yoelii	TAVVHQLKRKH

	(TRAP)	SEQ ID NO:141
V2.Pv-T1A	P. vivax	PAGDRADGQPAGDRAAAGQPAG SEQ ID NO:142
V2.ALV1.2	ALV-J	NQSWTMVSPINV SEQ ID NO:143
V2.ALV1.2	ALV-J	MIKNGTKRTAVTFGSV SEQ ID NO:144
V2.FMDV (142-160)	FMDV	PNLRGDLQVLAQKVARTLP SEQ ID NO:145
V2.FMDV (135-160)	FMDV	RYNRNAVPNLRGDL-QVLAQKVARTLP SEQ ID NO:146

Example 5: <u>Determination of 280:260 Absorbance Ratios</u>

Protein samples were diluted to a concentration of between 0.1 and 0.3 mg/mL using phosphate buffered saline (PBS), pH 7.4. The spectrophotometer was blanked, using PBS, and the absorbance of the protein sample measured at wavelengths of 260 nm and 280 nm. The absorbance value determined for a sample at 280 nm was then divided by the absorbance value determined for the same sample at 260 nm to achieve the 280:260 absorbance ratio for a given sample. The ratios obtained for several samples, including native particles (HBc 183), HBc particles truncated after residue position 149 (HBc 149), and several HBc chimers that are identified elsewhere herein, are shown below in Table 8.

Table 8

Particle	280/260 Absorbance Ratio
HBc183	0.84
HBc149	1.59
V2.Pf1	1.64
V2.Pf1+C150	1.5
V2.Pf1 + Pf/CS-UTC	1.54
V2.Pf1 + Pf/CS-UTC(C17A)	1.42

Example 6:   Cysteine at the C-terminus
              of Truncated HBc Particle

     A.   Addition of a Cysteine Residue
     to the C-terminus of Hybrid HBc Particles

     Using the polymerase chain reaction (PCR),
genes expressing hybrid HBc particles can be easily
mutated to introduce a cysteine or cysteine-
containing peptide to the C-terminus of HBc. For
example, a PCR oligonucleotide primer such as that of
SEQ ID NO:148 can be used, in concert with a suitable
second primer, to amplify a hybrid HBc gene and
incorporate a cysteine codon between codon V149 and
the stop codon.

     Hepatitis B core particles can be truncated
from residue position 183 (or 185, depending on viral
subtype) to 140 and retain the ability to assemble
into particulate virus-like particles. Many groups
have used particles truncated to amino acid 149

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because amino acid 150 represents the first arginine residue of the arginine-rich C-terminal domain.

To assess the ability of a single cysteine residue to stabilize HBc particles, a codon for a cysteine residue was inserted using techniques described before between the codon for HBc amino acid residue V149 and the termination codon of a chimer HBc molecule that contained the (NANP)$_4$ malarial B cell epitope inserted between residues 78 and 79 (referred to herein as V2.Pf1) to form the chimeric molecule and particle referred to as V2.Pf1+C. The thermal stability (at 37°C) of this chimer particle (V2.Pf1+C), as compared to a similar chimer particle lacking the inserted cysteine (V2.Pf1), was found to be dramatically increased, as is seen in Fig. 3.

It is noted that vectors and expression products that are prepared by addition of a cysteine to the C-terminus of a V2 construct are sometimes referred to herein as V16 vectors or expression products.

As can readily be seen in Fig. 3, the two particles started out similarly. However, after fourteen days at 37°C, the cysteine-containing particle exhibited fewer bands on the SDS gel, indicating enhanced stability as compared to the particle lacking the added Cys residue.

B.   Thermal Stability Protocol

Purified particles were diluted to a concentration of 1 mg/mL using 50 mM NaPO$_4$, pH 6.8 and sodium azide was added to a final concentration of 0.02% to prevent bacterial growth. Particles were incubated at 37° C and aliquots were taken at the time points indicated in the drawing description. Samples

were mixed with SDS-PAGE sample buffer (reducing) and run on 15% SDS-PAGE gels. Gels were stained using Coomassie Blue, and then analyzed.

Example 7:   Analytical Gel Filtration
                Analysis of Hybrid particles

Analytical gel filtration analysis of purified hybrid HBc particles was performed using a 25 mL Superose® 6 HR 10/30 chromatographic column (Amersham Pharmacia # 17-0537-01) and a BioCAD™ SPRINT Perfusion Chromatography System. The UV detector was set to monitor both wavelengths of 260 and 280 nm. The column was equilibrated with 3 column volumes (CV; about 75 mL) of buffer (50 mM $NaPO_4$, pH 6.8) at a flow rate of 0.75 mL/minute.

The particles to be analyzed were diluted to a concentration of 1 mg/mL using 50 mM $NaPO_4$, pH 6.8. 200 Microliters (μL) of the sample were then loaded onto a 200 μL loop and injected onto the column. The sample was eluted from the column with 50 mM $NaPO_4$, pH 6.8 at a flow rate of 0.75 mL/minute.

Particles containing C-terminal cysteine residues or similar particles free of such cysteines were analyzed using the above procedure. Integration of the 280 nm trace was carried out using BioCAD™ software (PerSeptive™) to provide the results in Table 9A, below.

Table 9A

Particle	Percent After Purification	
	Particulate	Non Particulate
V12.Pf1(C17A)	67	33
V12.Pf1(C17A) + C150 *	100	0
V12.Pf1 *	98	2
V2.Pf1+CfHBc74-87+C*	97.8	2.2
V2.Pf1+CfHBc74-87	80.7	19.3

* C-terminal cysteine-stabilized particles.

Purified particles were assayed for the percentage of particles and then incubated in aqueous solution at 37°C as discussed before. The compositions were assayed for stability after fourteen days of incubation. The results of this analysis are shown in Table 9B, below.

Table 9B

Particle Name	Percent Particles Following Incubations at 37°C (Days)	
	Zero	14
V12.Pf1 *	98	96
V12.Pf1(C17A)	67	63
V12.Pf1(C17A)+C150 *	100	98

* See the note to Table 9A.

Fig. 7 shows the results of a SDS-PAGE analysis of the particles of Table 9B at days zero, 7 and 14 following incubation at 37°C. Results of a densitometric analysis of that SDS-PAGE analysis are shown in Table 9C, below.

Table 9C

Particle	Percent Full Length Monomer Following Incubation at 37°C		
	Days		
	Zero	7	14
V12.Pf1 *	100	94	93
V12.Pf1(C17A)	100	13	1
V12.Pf1(C17A)+C150 *	100	83	63

* See the note to Table 9A.

The particles of Tables 9A-9C and control
particles that contained an added lysine residue
between HBc residues usually numbered 76 and 77
without [HBc150(K77)] and with a C-terminal Cys
residue [HBc150(K77) + C] were analyzed for
immunogenicity in BALB/c mice via intraperitoneal
injection using 20 μg of the respective particles in
phosphate buffered saline (pH 7.4) in the absence of
adjuvant, contrary to the results reported in Example
4. Sera were analyzed two weeks after immunization
using an ELISA with HBc particles (Anti-HBc.) or
(NANP)$_5$ synthetic peptide [Anti-(NANP)$_n$] as the solid
phase capture antigen. The results of this study are
shown in Table 9D, below

Table 9D

Particle	End Point Titer	
	Anti-HBc	Anti-(NANP)$_n$
V12.Pf1(C17A)	10,240	0
V12.Pf1 (C17A)+C150 *	10,240	2,560
V12.Pf1 *	10,240	10,240
HBc150(K77)	40,960	0

HBc150(K77)+C*	163,840	0

* See the note to Table 9A.

The data from this study are interpreted to mean that the C-terminal cysteine-stabilized particles are more stable immediately on production, as well as after incubation at 37°C for various time periods. The stabilized particles also exhibit enhanced immunogenicity, even in the absence of adjuvant. In addition, although particulate matter is present in the non-stabilized material such as V12.Pf1(C17A), there are no monomeric chimeric proteins after fourteen days of incubation and the material present does not induce antibodies toward the initially introduced heterologous B cell epitope sequence, here a malarial immunogen.

Example 8:   Cysteine Located Within a Peptide
             Fused to the C-terminus of an HBc Hybrid

Studies were conducted to determine if there were an absolute requirement for a cysteine residue to be the final amino acid of the HBc gene (as it is in wild type HBc) or if a cysteine could function internally in an introduced C-terminal sequence.

A peptide corresponding to a 20-residue universal T cell epitope, derived from the CS protein of the malarial parasite *Plasmodium falciparum*, which contains a cysteine at position 17 of the peptide or 342 of the CS protein, [Calvo-Calle et al., *J. Immunol.*, (1997) 159(3):p. 1362-1373], was fused to the C-terminus of a HBc chimer (V2.Pf1). This chimer contains the HBc sequence from position 1 through

position 149, with the *P. falciparum* B cell epitope (NANP)$_4$ inserted between amino acid residues 78 and 79. Domain I of this HBc construct thus contained residues 1-75; Domain II contained residues 76-85 with the (NANP)$_4$ epitope inserted between residues 78 and 79 (along with four residues comprising the restriction sites); Domain III contained residues 86-135; and Domain IV contained residues 136-149 plus the 20-residue *P. falciparum* T cell epitope and two residues from the EcoRI cloning site (GI).

This fused C-terminal peptide is 20 amino acid residues long (12 or 14 amino acids shorter than the wild type sequence, depending on virus subtype) and has a predicted pI value more than 8 pH units lower than the wild type sequence. To minimize potential stabilizing effects that may be contributed by amino acids other than the cysteine, a (similar) control construct was made, having an alanine instead of a cysteine at position 17 (see Table 10, below).

To enable simple assessment of the stabilizing effects of this sequence, the peptides were fused to the C-terminus of a particle previously shown to degrade readily at 37°C (V2.Pf1) to form the HBc chimers denominated V2.Pf1+Pf/CS-UTC and V2.Pf1+Pf/CS(326-345/C342A), respectively. The results of a thermal stability study over a 28 day time period (as discussed previously) are shown in Fig. 4.

The results of this study showed that the presence of the cysteine in the T cell epitope derived from the CS protein of *P. falciparum* was needed for particle stability in the time period studied, and that there was no absolute requirement that that cysteine be at the C-terminus of the

epitope. The table below shows the amino acid
sequences of C-terminal fusions with a cysteine or
alanine at position 17, relative to the native
sequence, which occurs in the wild type HBc protein.

Table 10

Source	Sequence	pI	Length	Cys Position	Cys Shift
Native	RRRGRSPRRRT- PSPRRRRSQSP- RRRRSQSRESQC SEQ ID NO:147	12.74	34	34	zero
Pf/CS-UTC	(GI)EYLNKIQNS- LSTEWSPCSVT SEQ ID NO:148	4.44	20	17	-15
Pf/CS- UTC(C17A)	(GI)EYLNKIQNS- LSTEWSPASVT SEQ ID NO:149	4.44	20	N/A	N/A

(GI) = residues added from cloning site.

Example 9: _P. Vivax_ HBc Chimers

Following the work discussed before on HBc
chimers containing _P. falciparum_ B cell and T cell
immunogens, similar work was carried out using
sequences from the _P. vivax_ CS protein. Exemplary
constructs are illustrated below in Table 11.

Table 11

P. vivax Immunogen Type	Malarial B Cell Immunogen (Between D78/P79)	CS-UTC (After V149)
Type-I	(DRAAGQPAG) SEQ ID NO:152 (DRADGQPAG) SEQ ID NO:153	YLDKVRATVGTEWTPCSVT SEQ ID NO:25
Type-II	(ANGAGNQPG) SEQ ID NO:154 (ANGAGDQPG) SEQ ID NO:155 (ANGADNQPG) SEQ ID NO:156 (ANGADDQPG) SEQ ID NO:157	YLDKVRATVGTEWTPCSVT SEQ ID NO:25
Type-III ('Vivax-like')	(APGANQEGGAA) SEQ ID NO:158	YLDKVRATVGTEWTPCSVT SEQ ID NO:25

To address the variability of the repeats, the following variant epitopes were used for insertion into HBc between amino acids 78 and 79:

1.   Type-I CS-repeat

PAGDRADGQPAGDRAAGQPAG (*P. vivax*-type 1A)--SEQ ID NO: 159. This form of the epitope failed to make a particle.

DRAAGQPAGDRADGQPAG (*P. vivax*-type 1B)-- SEQ ID NO: 150. This form of the epitope, containing flanking dipeptide cloning site remnants, successfully made a particle and is referred to as V2.PV-TIB. An immunogen for *P. vivax*-type I has been successfully cloned, expressed, purified, and its immunogenicity

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tested in mice. The results of that mouse study are shown in Table 12, hereinafter.

2. Type-II CS-repeat

For type-II, this work is complicated by the existence of four different forms of the type-II epitope. These forms contain either G or D at position 5, and either N or D at position 6 [Qari et al., *Mol. Biochem. Parasitol.*, (1992) 55(1-2):p. 105-113]. Hence, there are 4 different possible repeat sequences (GN, GD, DN, and DD) needed to maximize the possibility of success. The first, and preferred approach, is to prepare a single hybrid particle containing all four repeats, as shown below by underlines. This approach was successfully employed to address the variability in the type-I repeat. Each of these constructs contains flanking dipeptide cloning site remnants.

ANGAGNQPGANGAGDQPGANGADNQPGANGADDQPG

(*P. vivax*-type II -GN/GD/DN/DD)   SEQ ID NO: 151.

The above sequence has been cloned, expressed, and purified as a HBc chimer with no modification to the C-terminus.

The second approach was to prepare two hybrid particles, whereby each particle contained two of the variant epitopes (see below). This approach is less preferable because it requires either the use of a more complex expression system to direct the production of 'mixed' particles during expression, or the mixing of type-II particles following manufacture.

ANGA<u>GN</u>QPGANGA<u>GD</u>QPG   (*P. vivax*-type II-GN/GD)

SEQ ID NO: 160.

QANGA<u>DN</u>QPGANGA<u>DD</u>QPG   (*P. vivax*-type II-DN/DD)

SEQ ID NO: 161.

CGCGAATTCAAGCGAACGGCGCCGATAATCAGCCGGCGGGTGCA

(*P. vivax*-type IIB-ER1-wt-F)   SEQ ID NO: 162.

3.   <u>Type-III ('vivax-like') CS-repeat</u>

The third *P. vivax* CS-epitope, which is quite
different from the other two, is not associated with
amino acid variation (see below) [Qari et al.,
Lancet, 1993. 341(8848): p. 780-783]. This sequence
was cloned into the HBc expression system, and
hybrids were produced that contained flanking
dipeptide cloning site remnants.

APGANQEGGAAAPGANQEGGAA (*P.vivax*-type III)

SEQ ID NO: 163.

4.   <u>T cell Epitope at the C-terminus of HBc</u>

The insertion of the *P. vivax* Th epitope
(Pv-UTC; YLDKVRATVGTEWTPCSVT; SEQ ID NO:25) into HBc
and HBc hybrids was also performed using synthetic
DNA fragments (Synthetic Genetics, San Diego CA).
However, unlike B cell epitopes, which are inserted
into the immunodominant loop region of the HBc gene,
T cell epitopes are fused to the C-terminus of the
HBc gene. Previously discussed cloning vectors were
used for the insertion of both B and Th epitopes into
HBc. The particle expressing just the Pv-UTC at the
C-terminus has also been successfully made.

5. Combining B and T cell Epitopes
   in a Single Particle

   To combine B and Th epitopes into single
HBc constructs, PCR is used to amplify N-terminal HBc
fragments (residues 1-80, which contain the B cell
epitopes), and C-terminal HBc fragments (residues 81-
150, which contain the T cell epitopes). The
fragments are ligated together and amplified again by
PCR. Again, clones are verified by restriction
endonuclease mapping and automated DNA sequence
analysis (Lark Technologies, Houston TX). Details
are essentially the same as for *P. falciparum*.
Particles that contain each of the Type-I, -II and
-III B cell epitopes and variants as well as the Pv-
UTC, have been expressed and recovered.

Example 10: Relative Immunogenicities of HBc Chimers

   Relative immunogenicities of several HBc
chimer immunogens were compared in mice using the IFA
assay discussed previously. The results of those
studies using two dose immunization regimens as
before are shown below in Table 12.

Table 12

Immunogen	IFA titer	Protection	Citation
P.berghei (CS-1)	40,960	95%	A
P.yoelii (CS-3)	12,800	95%*	B
P.falciparum (CS-2)	1,200	NT	A
P.falciparum	5,200,000	NT	--

(V12.Pf3.1)

P.vivax (V2.PV-TIB)   160,000   NT   --

[A = Schodel et al., J. Exp. Med., 1994, 180:1037-1046. B = Schodel et al., *Behring Inst. Mitt.*, 1997(98): p. 114-119. NT = not tested. * = protection for greater than 3 months.]

As is seen from the above data, titers of 10^5-10^6 for *P. falciparum* were achieved using a contemplated chimeric immunogen; this compares to titers of only 10^4 for *P. berghei* and 10^3 for *P. falciparum* using the replacement technology of Schodel et al.

Mice were immunized with CS-2 or V12.Pf1 using 20 µg of particles on day zero and were boosted with 10 µg at four weeks. Mice immunized with particles from V12.Pf3 and V12.Pf3.1 were immunized using 20 µg of particles on day zero and were boosted with 10 µg at eight weeks using adjuvants as discussed before. Data showing the duration of the titers achieved are shown in Fig. 5, with data for use of V12.Pf3 particles being essentially identical to data with V12.Pf3.1 particles, and not shown.

Example 11: Relative HBc antigenicities

A series of studies was carried out to determine the relative antigenicities of several malarial HBc chimer particles toward two monoclonal antibodies (MoAb-3120 and MoAb-3105) as compared to native HBcAg (particle). These antibodies are specific to the loop region of HBc, and were the

gracious gift of the Immunology Institute, Tokyo, Japan. Studies were carried out using the chimers of Table 5 that contain malarial epitopes inserted into HBc particles at various positions as antigens in ELISA assays with the monoclonals as probes. The results of these studies (as end point dilutions) are shown below in Table 13A, 13B, and 13C, and illustrate the substantial lack of antigenicity of a contemplated chimer toward monoclonal antibodies that bind to the loop region, the primary immunogen, of HBc. Put differently, monoclonal antibodies that bind specifically to the loop region of HBc barely recognize a contemplated chimer, if at all.

Table 13A

Particle	Anti-MoAb-3120 End Point Dilution	Relative Antigenicity
HBcAg	625000	100
V12.Pf3	80000	12.8
V12.Pf3.1	20000	3.2
V12.Pf3.2	10000	1.6
V12.Pf3.3	10000	1.6
V12.Pf3.4	80000	12.8
V12.Pf3.5	40000	6.4
V12.Pf3.6	80000	12.8
V12.Pf3.8	80000	12.8
V12.Pf3.9	160000	25.6
V12.Pf3.10	10000	1.6
V12.Pf3.11	80000	12.8
V12.Pf3.12	80000	12.8

Table 13B

Anti-MoAb-3105

Particle	End Point Dilution
HBcAg	1,300,000
V2.Pf1 (78/79)	Zero
V12.Pf1 (78/79)	Zero
V12.Pf3 (78/79)	Zero
V1.Pf1 (77/78)	Zero
V13.Pf1	1,300,000

An insertion into several sites in the immunodominant loop (including positions 77-78 or 78-79) totally eliminates binding of MoAb-3105. V13 is an insertion between residues 129 and 130 and is used as a control because the native HBc immunodominant loop remains intact in this construct.

Table 13C

Anti-MoAb-3120

Particle	End Point Dilution
77/78 V1.Pf1	102,400
78/79 V2.Pf1	400
HBcAg	409,600

These data show that insertion between residues 78 and 79 causes a more drastic reduction in anti-MoAb-3120 binding as compared with insertion between residues 77 and 78.

Example 12:   Preparation of Vector for Preparation
of HBc Particles for Use in Humans

A.   Preparation of Vector V17Pf3.1

To manufacture the particle V12.Pf3.1 in a
manner suitable for human administration, it was
necessary to express the particle using an expression
system that did not require the use of ampicillin to
ensure plasmid maintenance.   To achieve this, the
gene coding for the particle, along with the
necessary upstream regulatory sequences, was inserted
into a new plasmid that utilizes kanamycin as the
selectable marker.   The new plasmid (V17.Pf3.1) was
synthesized using a two step cloning procedure:

Step 1: The plasmid pKK223-3N-V12 was
digested with the restriction enzymes BamHI and
HindIII to yield two DNA fragments of 801 and 4869
bp.   In addition, the commercially available plasmid
pREP4 (Qiagen) was cut with BglII and HindIII to
yield two fragments of 320 bp and 3420 bp.   The 3420
bp and 801 bp fragments were ligated to create
plasmid V17. (It is noted that BglII and BamHI·
digested DNAs can be ligated by virtue of their
common 'overhang' sequences, although neither BglII
or BamHI can cut the resultant fragment).   The V17
plasmid, therefore, contains the HBc149 gene,
complete with Pf-UTC sequence fused to the C-
terminus, and EcoRI and SacI restriction sites in the
immunodominant loop region to enable insertion of
epitopes between D78 and P79 of the HBc gene.

Step 2: The second step was to insert the
Pf3.1 version of the Pf CS-repeat epitope into the
immunodominant loop region of the gene.   This was
achieved by digesting V17 with SacI and EcoRI to

yield 15 bp and 4206 bp DNA fragments. Annealed
oligonucleotides encoding the Pf3.1 epitope were
ligated with the 4206 bp fragment to yield V17.Pf3.1,
a 4275 base pair plasmid. In addition to the gene
that encodes the 195 amino acid malaria vaccine
candidate, this plasmid contains a gene for the lac
repressor (lac I) to force any gene under lac
promoter control to be fully repressed until induced
by isopropylthiogalactoside (IPTG). It also has a
kanamycin resistance gene to permit positive
selection via the addition of kanamycin to culture
media. The plasmid has the replication origin of
pACYC 184 and is not considered to be a high copy
number plasmid.

The locations of the genes of interest are:

Gene	Start	Stop	Amino Acids	Molecular Weight (kDa)
Lac I	2128	3087	319	34.1
V17.Pf3.1	281	868	195	21.7
KmR	4259	3465	264	29.1

A suitable host for V17.Pf3.1 is *E. coli*
BLR, a rec A derivative of *E.coli* BL21, and a common
strain used for the production of recombinant
proteins (available for purchase from Novagen). *E.
coli* BLR was selected as a host organism for
expression because of its increased genetic
stability, as well as its ability to produce

assembled particles in soluble form (not in inclusion bodies).

B. Expression of Particles
Using Plasmid V17.Pf3.1

E.coli (Strain BLR) containing the V17.Pf3.1 plasmid were streaked onto an LB agar plate supplemented with 25 μg/mL kanamycin and 10 μg/mL tetracycline, then incubated at 37°C for 16-20 hours. A single colony was then used to inoculate 3 mL of TB-Phy medium in a sterile culture tube, supplemented with 25 μg/mL kanamycin. The tube was incubated overnight (about 18 hours) on a shaker at 37°C and about 200 rpm.

The following morning, 100 mL of TB-Phy medium was warmed to 37°C. One mL of the overnight culture was removed and used to inoculate the flask, which was then incubated on a shaker at 37°C at about 200 rpm for six hours.

The fermentor (Biostat™ UE20) was inoculated with 100 mL of inoculum with the fermentor conditions set as follows:

Agitation	400 rpm
Temperature	37°C
Aeration	air, 10 liters per minute
pH	7.0, uncontrolled

The A_{600} value was measured for the first sample, and for samples every 20-30 minutes thereafter to monitor A_{600}. An IPTG solution was prepared by dissolving 62 mg IPTG in 10-15 mL water. When the A_{600} value reached 0.5, the filter-

sterilized IPTG solution was aseptically added to the fermentor through a syringe. The incubation was continued until next day (e.g. about another 10-24 hours).

At 14 hours after induction, the fermentor temperature was set to 15°C. Harvesting of cells was started by centrifugation in a Beckman® J2-MC centrifuge with following conditions:

Rotor	JA10
Speed	7,500 rpm
Temperature	4°C
Time	9 minutes

The cells were harvested by freezing into liquid nitrogen.

C. Purification of Particles
Expressed by Vector V17.Pf3.1/BLR

The biomass of harvested cells was resuspended in 50 mM sodium phosphate, pH 6.8, and lysed using a French Pressure cell at 16,000 psi. The cell debris was removed by centrifugation using a Beckman® J2-MC centrifuge and the following conditions.

Rotor:	JA20
Speed:	15,000 rpm
Temperature:	4°C
Time:	30 minutes.

The volume of the resultant supernatant was measured and 277 g/L of solid ammonium sulfate were slowly added to the supernatant. The mixture was

stirred at 4°C for 30 minutes. The solution was centrifuged in Beckman® J2-MC centrifuge with the following conditions.

Rotor: JA20
Speed: 15,000 rpm
Temperature: 4°C
Time: 30 minutes

The precipitate was then resuspended in a minimal volume of 50 mM sodium phosphate buffer and then dialyzed against the same buffer for one hour with stirring. The dialyzed solution was centrifuged in Beckman® J2-MC centrifuge with the following conditions.

Rotor: JA20
Speed: 15,000 rpm
Temperature: 4°C
Time: 15 minutes

The supernatant was recovered and then subjected to gel filtration chromatography.

System: Pharmacia Biotech AKTA™ Explorer
Buffer B (elution solvent): 50 mM Sodium phosphate buffer (pH 6.8).
Column: Millipore Vantage™ VL44 x 1000 column (44 mm diameter, 1000 mm height, Catalog No.: 96441000)
Resin: 1.5 liter Sepharose® CL-4B manufactured by Pharmacia
Detector: UV at 210, 254 and 280 nm.

Fraction: 15 mL

The column was eluted with buffer B at 2 mL per minute. Particle-containing fractions were identified using SDS-PAGE and pooled. The salt concentration of the pooled material was adjusted to 5M by adding sodium chloride.

Hydrophobic Interaction Chromatography:

System: Pharmacia® Biotech AKTA™ Explorer
          (System No.: 18111241 001152,
          University of Iowa ID No.: 540833.)

Buffer A: 50 mM sodium phosphate buffer
            (pH 6.8) + 5 M NaCl. (The buffer
            was degassed for 30 minutes
            daily, before use.)

Buffer B (elution solvent):   50 mM sodium
            phosphate buffer (pH 6.8). (The
            buffer was degassed for 30 minutes
            daily, before use.)

Hydrophobic Interaction Chromatography using ToyoPearl® ether 650 resin

Column: Millipore Vantage™ VL44 x 250
          column (44 mm diameter, 250 mm
          height, Catalog No.: 96440250)
Resin: 200 mL Toyopearl® ether 650 HIC
          resin, manufactured by Tosohaas
Detector: UV at 210, 254, and 280 nm

Fraction: 15 mL

The column was equilibrated with 5 column
volumes (CV) of buffer A for a one hour time prior to
starting purification, using a flow rate of 20
mL/minute. The retentate containing 5 M salt was
then loaded at a rate of 20 mL/minute. The column
was washed with 2 CV of buffer A, washed with 2 CV of
10% buffer B, eluted with 3 CV of 40% buffer B, and
(finally eluted) with 100 % buffer B. Fractions were
completely analyzed for proteins of interest by SDS
PAGE analysis. Pure fractions were combined
together, and a protein estimation using a Bradford
assay was carried out.

Hydrophobic Interaction Chromatography using butyl
resin

Column: Millipore Vantage™ VL44 x 250
          column (44 mm diameter, 250 mm
          height, Catalog No.: 96440250)
Resin: 200 mL Toyopearl® Butyl 650-S HIC
          resin, manufactured by Tosohaas
Detector: UV at 210, 254 and 280 nm
Fraction: 15 mL

The column was equilibrated with 5 column
volumes (CV) of 40% buffer B for one hour prior to
starting purification, using a flow rate of 20
ml/min. The combined fractions from ether HIC were
loaded at a rate of 20 mL/minute. The column was
washed with 2 CV of 40% buffer B, washed with 2 CV
90% B, and eluted with 4 CV of WFI.

Fractions were analyzed for protein of interest by SDS PAGE analysis. Pure fractions were combined together

Hydroxyapatite Column Chromatography

Column: Millipore Vantage™ VL16 x 250
column (16 mm diameter, 250 mm
height, Catalog No.: 96160250)
Resin: 20ml Ceramic Hydroxyapatite (Catalog No. 158-2200)
Detector: UV at 215, 254 and 280 nm
Fraction: 15 mL

The column was equilibrated with 5 column volumes (CV) of 20 mM sodium phosphate buffer, flow rate: 5 mL/min. Load combined fractions eluted from butyl HIC at 5 mL/min. Wash the column with 20 mM sodium phosphate buffer until A280 drops to baseline. Fractions were analyzed for protein of interest by SDS PAGE analysis. Pure fractions were combined together.

Desalting

Column: Prepacked desalting column, HiPrep™ 26/10, Pharmacia
Resin: 20 mL Ceramic Hydroxyapatite (Catalog No. 158-2200)
Detector: UV at 215, 254 and 280 nm
Fraction: 15 mL

The column was equilibrated with 5 CV of 15 mM Acetate Buffer, pH 6.0. The pooled fractions from the hydroxyapatite column were loaded onto the column, and then eluted with 15 mM Acetate Buffer, pH 6.0, at a flow rate of 20 mL/min. Fractions were analyzed for protein of interest by SDS PAGE analysis. Pure fractions were combined together, and protein estimation was carried out using a Bradford assay. The pure fraction was assayed for endotoxin level, and finally passed through a 0.22-micron filter for terminal filtration.

Example 13: Preparation of Vectors to Express Particles with a Cysteine Residue Prior to C-Terminal Fused Epitope

To prepare particles with a single cysteine after V149 of the HBc gene, followed by a T cell epitope, a PCR primer was synthesized (SEQ ID NO:165). This primer, in conjunction with HBc149/NcoI-F (SEQ ID No:29), was used to amplify the HBc gene to produce a version of HBc having a single cysteine codon introduced directly after V149, as well as EcoRI and HindIII restriction sites (after the introduced cysteine). The 478 bp PCR product was cut with NcoI and HindIII and cloned into pKK223-3N.

SEQ ID No.: 164

```
                          C   V   V   T   T   E   P
5' GCAAGCTTACTATTGAATTCCGCAAACAACAGTAGTCTCCGG
       HindIII       EcoRI
```

SEQ ID NO:165

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The resultant plasmid was then cut with
EcoRI and HindIII, and the annealed oligonucleotides
coding for the Pf-UTC (Pf/CS326-345; SEQ ID NOs: 80
and 81) ligated into the plasmid. This plasmid was
then used as the template in a PCR reaction, along
with the primers HBc-P79/SacI-F (SEQ ID NO:35) and
Pf/CS-UTC(C17A) (SEQ ID NO:104) the resultant PCR
product (307 bp) coded for amino acids 79 through 149
of HBc, followed by the introduced cysteine, followed
by the Pf/CS-UTC having the C17A mutation [Pf/CS-
UTC(C17A)], and flanked by SacI (5') and HindIII (3')
restriction sites. This fragment was cut with SacI
and HindIII and ligated with the plasmid V2.Pf1
(encoding the malarial (NANP)$_4$ epitope) that had been
cut with the same two enzymes.

The resultant gene codes for a 190 amino
acid HBc chimera having (NANP)$_4$ inserted between
amino acids 78 and 79 of HBc, (flanked by the Gly-Ile
and Glu-Leu sequences derived from the EcoRI and SacI
restriction sites respectively) and the C17A version
of the Pf/CS-UTC at the C terminus. The single
cysteine was therefore located between V149 of HBc
and the Gly-Ile linker sequence (derived from the
EcoRI restriction site) located prior to the first
amino acids of the Pf/CS-UTC(C17A) T cell epitope
(see SEQ ID NO:167).

This hybrid particle was expressed, purified and
analyzed for stability by incubating at 37°C for
several weeks. The stability of this particle
(V12.Pf1(C17A)C150) was compared to V12.Pf1, with the
only difference between the two particles being the
position of the cysteine residue. For V12.Pf1 the
cysteine is followed by 3 amino acids (SVT) at the C-
terminus of the protein (SEQ ID No: 166), whereas for

V12.Pf1(C17A)C150 the cysteine is followed by 22
additional amino acid residues (SEQ ID No: 167).

V12.Pf1

    TTVV   GI EYLNKIQNSLSTEWSP<u>C</u>SVT   SEQ ID No:166

V12.Pf1(C17A)C150

    TTVV <u>C</u> GI EYLNKIQNSLSTEWSP<u>A</u>SVT   SEQ ID No:167

      The effect of inserting the cysteine
residue between HBc and the T cell epitope
(V12.Pf1(C17A)C150) was to create a particle that was
significantly more stable than a similar particle
without the C terminal cysteine (V12.Pf1(C17A)).
This was evident from the fact that unlike
V12.Pf1(C17A), V12.Pf1(C17A)C150 could be easily
purified without a significant degree of degradation
of monomers (compare T=0 for these particles in Figs.
4 and 7); and further, V12.Pf1(C17A)C150 was
significantly more stable than V12.Pf1(C17A)
following incubation at 37°C. After 14 days at 37°C,
V12.Pf1(C17A) monomers are totally degraded (Fig. 4),
whereas V12.Pf1(C17A)C150 monomers are only partially
degraded (Fig. 7).

      It was apparent that V12.Pf1(C17A)C150 was
not as stable V12.Pf1 (Fig. 7). These data indicate
that the stabilizing effects of a single C-terminal
cysteine residue are most effective when placed at or
near, e.g. within five residues of, the C-terminus of
the HBc chimer.

Example 14: Preparation of Vectors to Express
           Particles with a Cysteine Residue at
           <u>the C-Terminus of a Fused Epitope</u>

To further investigate whether terminal cysteine residues could elicit stabilizing effects at positions other than 150, a Th epitope from the hepatitis B core protein (amino acid residues 74-87) was fused to the C-terminus of HBc containing a malarial epitope [(NANP)$_4$] in the immunodominant loop. This Th epitope does not contain a cysteine residue, so a Cys residue was added at the C-terminus (underlined "C"). The control contained the same fused epitope lacking the cysteine.

These particles were made by combining V2.Pf1 with V7.HBc74-87 (and V7.HBc74-87+C) to form the desired vector. The V7 construct was PCR amplified with the HBc-P79/SacI-F primer (SEQ ID NO: 35) and pKK223-2/4515-32-R (SEQ ID NO:36). The product was cut with SacI and HindIII, and the SacI/HindIII fragment was ligated into V2.Pf1 cut with the same enzymes.

Table 14, below, shows the amino acid sequences of C-terminal fusions HBc(74-87) and HBc(74-87) + C, relative to the native sequence that occurs in the wild type HBc protein, as well as the and the HBc149 + C particle. "Cys shift" is the position of the introduced cysteine relative to its location in the wild type protein, where it is the last residue (position 183).

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Table 14

Source	Sequence	PI	Length	Cys Position	Cys Shift
Native	RRRGRSPRRRT-PSPRRRRSQSP-RRRRSQSRESQ<u>C</u> SEQ ID NO:147	12.74	34	34	Zero
HBc(74-87)	GIVNLEDPAS-RDLVV<u>S</u> SEQ ID NO:182	3.78	16	N/A	N/A
HBc(74-87)+C	GIVNLEDPAS-RDLVVS<u>C</u> SEQ ID NO:183	3.78	16	16	-17
HBc-149+C	<u>C</u>	N/A	1	1	-33

Example 15:   Comparative Immunogenicities in Monkeys

The comparative immunogenicity of the particles expressed by V12.Pf3.1, formulated with either Seppic™ ISA-720 (Seppic Inc., Paris, France), Alhydrogel™ (Superfos, Denmark) as adjuvants, or unformulated (saline), was studied in Cynomolgus monkeys.

The Seppic™ ISA-720 formulation was prepared according to the manufacturers directions. Briefly, the ISA-720 and V12.Pf3.1 particles were mixed at 70:30 (w/w) ratio and vortexed, using a bench top vortexer, set at maximum power, for 1 minute. The Alhydrogel™ formulation was prepared using an 8-fold excess of Alhydrogel™ (by weight) over V12.Pf3.1 particles, which was shown to be physically bound to the Alhydrogel™ prior to immunization.

Groups of two monkeys (one male and one female) were immunized with 20 µg V12.Pf3.1 particles as immunogen via the intramuscular route. Animals were bled on days 0, 21, 42, 56 and 70, and sera analyzed for titers of anti-NANP antibody using an ELISA. The results, shown in Table 15, below,

demonstrate the extremely high immunogenicity of
V12.Pf3.1 particles when formulated with Seppic™ ISA-
720 versus Alhydrogel™-formulated or unformulated
material. The kinetics of the antibody response were
more rapid when Seppic™ ISA-720 was used as the
adjuvant, and the end-point titers were more than
100- and 1000-fold higher than for Alhydrogel™ and
saline respectively.

Table 15

Adjuvant	Antibody Titers at Stated Time (Days)				
	Zero	21	42	56	70
Saline	Zero	40	240	1,200	640
Anhydrogel™	Zero	2,880	1920	11,500	6400
Seppic™ ISA-720	Zero	81,920	348,160	26,000,000	1,920,000

Example 16: T Cell Activation

Mice were immunized twice with V12.Pf3.1
particles in Seppic™ Montanide™ ISA-720. Spleen
cells were removed and stimulated in the presence of
various peptides. 10^6 cells were incubated for 3 days
in the presence of peptides: UTC (universal T epitope
from *P. falciparum*; Seq IN NO: 120), p85-100 peptide
corresponding to HBc 85-100, NANP (B-cell epitope
from V12.Pf3.1; NANPNVDP(NANP)$_3$,SEQ ID NO:22) in the
presence of *Staphylococcal* enterotoxin B (SEB), or
tissue culture medium (unstim). Interferon gamma
production after 3 days was determined by ELISA.

The results shown in Table 16, below, indicate that immunizing with V12.Pf3.1 induces T-cells that recognize the UTC component of the protein, and drives them to a Th1 type response.

Table 16

Immunogen	IFN-γ (pg/ml)	S.D.*
UTC	1600	750
p85-100	350	30
NANPNVDP (NANP)₃ SEQ ID NO:22	370	50
SEB	4300 ·	ND**
unstim	900	1100

* S.D. = Standard Deviation
** ND = Not Done

Each of the patents and articles cited herein is incorporated by reference. The use of the article "a" or "an" is intended to include one or more.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.